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Lipid polymer hybrid nanoparticles as targeted drug delivery system for melanoma treatment

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Abstract: Our understanding of the biologic effects (including toxicity) of nanomaterials are incomplete. In vivo animal studies remain the gold standard; however, widespread testing remains impractical, and the development of in vitro assays that correlate with in vivo activity has proven challenging. Here, we demonstrate the feasibility of analysing in vitro nanomaterial activity in a generalizable, systematic fashion. We assessed nanoparticle effects in a multidimensional manner, using multiple cell types and multiple assays that reflect different aspects of cellular physiology. Hierarchical clustering of these data identifies nanomaterials with similar patterns of biologic activity across a broad sampling of cellular contexts, as opposed to extrapolating from results of a single in vitro assay. We show that this approach yields robust and detailed structure–activity relationships. Furthermore, a subset of nanoparticles was tested in mice, and nanoparticles with similar activity profiles in vitro exert similar effects on monocyte number in vivo. This data suggests a strategy of multidimensional characterization of nanomaterials in vitro that can inform the design of novel nanomaterials and guide studies of in vivo activity.

Keywords: *Lipid polymer hybrid nanoparticles; melanoma cancer; nanocarriers; vitamin D receptors; bioactivity; targeted drug delivery system.*

I. Introduction

Melanoma occur as a result of genetic alterations in the cell which produce the pigment known as melanocytes[1]. Although melanoma accounts only 4 percent but it is responsible for highest number of deaths related to skin cancer[2]. Till date, the most commonly utilized treatment for melanoma cancer is chemotherapy, but it may also contain very serious side-effects on healthy cells of body, lesser bio-availability, poor selectivity on the way to tumor[3]. Another problem is that when chemotherapeutic drugs are utilized for the extended period of time, it results in developing MDR (multidrug resistance)[4]. If the treatment target just tumor cells, then side effects would be minimized. In case of melanoma, it shows very less response rate towards the conventional form of therapies that are present[5]. Nanocarries have been extensively utilized for delivering drug to targeted site of action which results in higher therapeutic efficiency[6]. The size of nanoparticles (NPs) ranges from the one to thousand nm (nanometers) in diameter. The diverse physio-chemical proper-

ties of nanoparticles e.g. size and of NPs, surface-area and surface charge of NPs give them benefit over current anti-cancerous treatments[7].

The most commonly utilized nanocarriers comprise of polymeric drug delivery system (DDS) and vesicular system based on the lipid[8]. Polymeric NPs show tremendous medicine loading and stability but less bio-compatibility, but in comparison, liposomes display very good biocompatibility but they face the complications of leakage of drug[9]. In order to overcome the limitations of NPs and liposomes, a new class of drug delivery system known as LPNPs (lipid polymer hybrid nanoparticles) has been established which contain properties of both liposomes and polymeric molecules which comprises of 3 parts, a bio-degradable polymeric core which is hydrophobic in nature and contain hydrophobic drug, and in order to enhance the system biocompatibility a single layer of phosphor-lipids which surround the core is present and then in order to rise the systemic circulation life-time and the stability, a hydrophilic polymer layer present outside the lipid[10]. In order to develop the lipid polymer hybrid nanoparticles, countless polymers have been designed which include PLGA (poly lactic co glycolic acid), PLA (polylactic acid), PbA (poly β amino ester and chitosan, these polymers are extremely bio-degradable and have high compatibility[11]. Like numerous varieties of nanocarrier, polymer lipid nanoparticles can also couple with targeting moieties so that the drug delivers precisely to the cells of tumour and tumour vasculature[12].

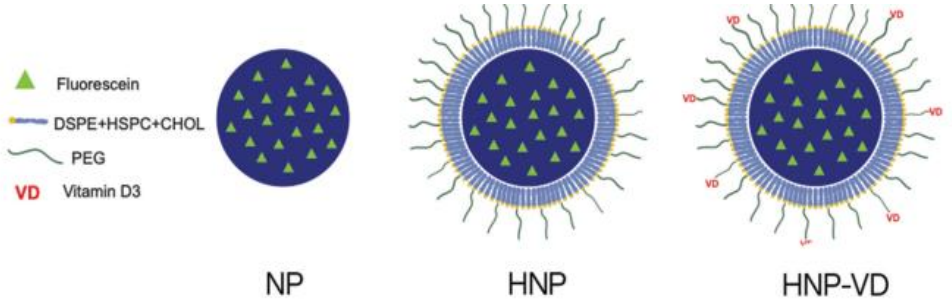

Figure 1. PLGA NP (nanoparticles), HNP (hybrid lipid polymer nanoparticles), HNP-VD (vitamin D3 functionalized hybrid lipid polymer nanoparticles)

These hybrid lipid polymer NPs have several benefits e.g., circulate for extensive period of time, higher capacity of drug loading, higher stability and property of controlled release[13]. PLGA (poly lactic co glycolic acid) nanoparticles are widely utilized for the delivery of therapeutic drugs due to their capability of maintain the drug for extended period of time, their compatibility[14]. These hybrid particles comprise of the poly lactic co glycolic acid (PLGA) as core and 2 lipids DSPE-PEG and HSPC (hydrogenated-soy phosphatidylcholine) form the shell which basically surround the particle and among lipids, the molecule of cholesterol play very important part in stabilizing the membrane[15]. HNPs (hybrid NP) were manufactured with folic acid in order to specifically delivering the paclitaxel against the cervical cancerous cells[16]. In order to enhance efficacy of hybrid nanoparticles, it was first time these hybrid NPs are basically coupled with ligand such as vitamin D which comprise of 2 main forms (Vitamin D2 and vitamin D3)[17]. Studies have indicated that vitamin D receptor (VDR) is present in body including skin and it is also located in melanoma cell[18]. The activated form of VD is the vitamin D3 (1,25 di-hydroxy

vitamin D3) which employs its effect on targeted tissue by activating the VDR[19]. This was first time in which HNP-VD (lipid polymer hybrid NPs with functionalized VD3) was synthesized in order to target the receptors of vitamin D and enhance the cell internalization and in this VD3 covalently attached to DSPE-PEG2000[20].

2. Material and Methods

Poly lactic co glycolic acid (PLGA 50:50) was donated by PURAC (Germany). The solvents EtAc (ethyl acetate), dichloro-methane (DCM) and dimethyl-sulfoxide (DMSO) were bought from Merck (Germany). Poly vinyl -alcohol (PVA), fluorescein, trehalose, cholesterol (CHOL), iron-III-chloride hexahydrate and ammonium thiocyanate were attained from Sigma-Aldrich (Germany). Chloroform was attained from Fisher Scientific in USA. For the synthesis of the HNPs, hydrogenated soy phosphatidyl-choline (HSPC), 1,2-disteroyl-sn-glycero-3-phosphaethanolamine-N-[succinyl(polyethylene glycol)-2000 (DSPE-PEG2000) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[cholecalciferol(polyethylene glycol)-2000] (DSPE-PEG2000-VD) were bought from Nano soft Polymers. In addition, all reagents for cell culture were bought from Gibco/Invitrogen (Life Technologies - Carlsbad, CA, United States).

2.1. Synthesis of PLGA nanoparticles

The manufacturing of polymeric nanoparticles, lipid-polymer hybrid nanoparticles and functionalized lipid polymer hybrid nanoparticles (Figure 1) were carried out according to the following procedures [21]. The manufacturing of the PLGA NPs was carried out by means of the solvent evaporation emulsification method. For individual sample, 50 mg of PLGA were evaluated and dissolved in 1 mL of a EtAc: DMSO (3:1) solution. Samples containing fluorescein and Nile-red were prepared by thawing 1.25 mg and 0.5 mg in the solvent phase, respectively. 2 aqueous solutions of PVA (0.3 and 3.0% w/v) were set and 25 mL of the 0.3% (w/v) solution was shifted into a 50 mL beaker and agitated at 250 rpm. 2 mL of the 3.0% (w/v) solution was further added to a 15 mL centrifuge tube. Subsequently, the PLGA solution was added dropwise to the centrifuge tube under forceful vortexing. Once the whole volume was added, 30 seconds of extra vortexing was conducted. Afterward, the centrifuge tube was placed on an ice bath and transferred to a probe ultra-sonicator for 30 seconds (60% amplitude and 8 W). This process (vortex 30 s/ultra sonicator 30 s) was repeated 3 times. Finally, the dispersion was moved to the 0.3% (w/v) PVA solution where the nanoparticles were permitted to harden for 12 h. NPs collection was led by transferring the dispersion to an Oak Ridge tube and centrifuging at 25,000 rcf for 60 minutes. After, the supernatant was wasted, and the particles were re-suspended in deionized water. This washing step was repeated 3 times. The NPs were then re-suspended in a trehalose: polymer (1:2) solution, frozen in liquid N2 and lyophilized for 72 hours.

2.2. Synthesis of hybrid lipid-polymer nanoparticles (HNPs)

HNPs were manufactured using the gentle hydration method. This method contains of the formation of a lipid thin film in a round bottom flask followed by the hydration of this layer by a dispersion of NPs. In this work, the hybrid NPs were

formed with and deprived of the presence of targeting ligands. Briefly, a mixture of lipids HSPC: CHOL:DSPE-PEG-2000 (2:1:0.1 molar ratio) was dissolved in 5 mL of CHCl3. Then, the solvent was evaporated by means of a rotary evaporator, producing a thin lipid-film on the wall of the round bottom flask. The NPs formerly prepared were re-suspended in 5 mL of deionized water (6 mg/mL), added to the round bottom flask containing the lipid film at 1 mL/min and sonicated for 10 min. The round bottom flask was transferred to a stirring plate and the HNPs were leftward to self-assemble for 30 min under mild stirring and then lyophilized. The HNPs containing the targeting ligand (HNP-VD) were set using the same method, however, the combination of lipids was substituted by HSPC: CHOL: DSPE PEG2000: DSPE PEG2000-VD (2:1:0.08:0.02 molar ratio). Furthermore, aiming to enhance the amount of lipids utilized in the synthesis, HNPs were ready by means of the following lipid: NPs ratios: 1:1, 1:10, 1:20 and 1:25 (w/w). Table 1 show amount used for various samples.

Table 1. Equivalents utilized in formation of HNPs for various lipid: NPs ratio.

Mass ratio (Lipid:NPs)	Molar ratio	M_w (g/mol)	mg	mmol
1:1				
HSPC	2	785	20.8	0.02650
CHOL	1	387	5.1	0.01320
DSPE-PEG_{2000}	0.1	2993	4.1	0.00140
1:10				
HSPC	2	785	1.92	0.00240
CHOL	1	387	0.52	0.00130
DSPE-PEG_{2000}	0.1	2993	0.40	0.00010
1:20				
HSPC	2	785	1.06	0.00130
CHOL	1	387	0.27	0.00070
DSPE-PEG_{2000}	0.1	2993	0.20	0.00007
1:25				
HSPC	2	785	0.70	0.00090
CHOL	1	387	0.20	0.00040
DSPE-PEG_{2000}	0.1	2993	0.10	0.00004

2.3. Characterization of the nanoparticles

The NPs and HNPs were categorised by FEG-SEM (field emission scanning electron microscopy), DLS (dynamic light scattering) and zeta potential. Microscopy investigations were achieved on the equipment FeiVR Inspect F-50. The images were gotten applying a drop of an aqueous solution of NPs or HNPs (100 mg/mL) directly to the top of the stub. Next, the samples were exposed to gold sputtering for 80s. The parameters for the imagining of particles were distance of 7–8 millimetre's, 20 kilovolt and magnification of 70,000–300,000. The determination of the hydrodynamic diameter, achieved by DLS, along with the zeta potential analysis, were conducted on the ZetaSizer Nano series equipment from Malvern, England.

2.4. Quantification of lipid surface coverage

The process of quantification of lipids on the surface of HNPs, defined, is based on 2 analyses.1st, a colorimetric assay was utilized in which the creation of complexes of phosphor-lipids with ammonium ferro-thiocyanate are quantified. Another, a H-NMR analysis is made to enumerate the amount of DSPE-PEG2000 present on the surface of the HNPs from the proton absorptions of lactate units (5.19 parts per million), glycolate units (4.91 parts per million) and ethylene oxide units (3.51 parts per million). The primary method was applied by thawing 2 mg of HNPs formed with dissimilar polymer/lipid ratios (1:1, 1:10, 1:20, 1:25 w/w) in CHCl3. The resultant solution was diverse with 2 mL of the ammonium ferro-thiocyanate solution and then vortexed (60 s) to form the complex. The subsequent mixture was centrifuged at small speed and the absorbance of HSPC/AF complexes were measured at the wavelength of 471 nm. The amount of lipids was considered by means of a standard curve beforehand set with the similar lipid composition of the shell of the HNPs. The typical solution of ammonium ferro-thiocyanate was set according to Stewart [29]. Momentarily, 27.03 g of iron-III-chloride hexahydrate and 30.4 g of ammonium thiocyanate were dissolved in 1 L of deionized water. Then, a 2:1:1 (molar ratio) combination of HSPC, CHOL and DSPE-PEG2000 was thawed in 100 mL CHCl3. Volumes between 0.1 and 1.0 mL of this solution were further added to 2 mL of the ammonium ferro-thiocyanate solution in a centrifuge tube and sufficient CHCl3 was further added to bring the ending volume to 4 ml. The system was vortexed for 1-minute, the organic-phase detached using a Pasteur pipette and at that moment added to a cuvette for absorbance study through UV/ Vis (wavelength 471 nm). From the values attained, the quantity of lipids per milligram of HNPs, the number of lipids per HNPs and the % of coating of HNPs can be calculated by Eqs.1–3, respectively.

$$V_1 = \frac{M_L / Mw_L}{M_{NPs}} \quad (1)$$

$$V_2 = V_1 N_A V_{NPs} \rho_{PLGA} \quad (2)$$

$$V_3 = \frac{V_2 S_L \times 100\%}{S_i} \quad (3)$$

Where V1 is the quantity of moles of lipids per mg of HNP, V2 is the amount of lipids per HNP and V3 is the % of coating of the HNP. ML is the mass of lipids utilized in the preparing the HNP, MNPs is the mass of nanoparticles examined, MwL is the molecular weight of lipids, VNPs is the individual volume of individually NP, ρPLGA is the density of PLGA and NA is the Avogadro-number. Si is the surface area of a single NP and SL is the area of the polar portion of the HSPC and DSPE-PEG2000 molecules, are assumed by 0.694 nm2 and 1.240 nm2, correspondingly.

2.5. Drug release study

In this work, the release of the perfect drug fluorescein from NPs, HNPs and HNP-VDs were achieved in triplicate by means of the dialysis method for 6 days. For individual experiment, 5 mg of sample were distributed in 1 mL of the release medium (PBS, pH 7.4, 0.01 M) and positioned in contact with the dialysis membranes (Mw cut-off =14,000 Dalton) in the Franz cell donor section (Automated Franz Cells –

Microette Plus, Hanson Research Corporation, USA). The receiver section, where 7 mL of the release medium were located, was sustained under constant moving at a temperature of 320C. At predetermined intermissions (0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120 and 144 h), 1 mL aliquot was with-drawn from the recipient medium and the same volume of PBS further added to preserve the infinite dilution condition. Examination of samples taken at diverse times were performed by spectrofluorimetric in a plate reader (Molecular Devices – SpectraMax M2e). To attain the encapsulation efficiency (EE) and drug loading efficiency, the methodology presented was utilized and their values can be considered according to Eqs. 4 and 5. Momentarily, 2 mg of fluorescein loaded nanoparticles were dissolved in 1 mL of aceto-nitrile and then added to 10 mL of PBS in order to disperse the PLGA from the fluorescein. Then, the PLGA was sieved through a cellulose filter (pore size: 0.2 mm) and the solution examined by UV/Vis at a fixed wavelength 490 nm. The concentration of fluorescein present in the examined nanoparticles was then considered from a calibration curve.

$$EE = \frac{\text{weight of drug in NPs} \times 100\%}{\text{weight of drug added}} \quad (4)$$

$$DL = \frac{\text{weight of drug in NPs} \times 100\%}{\text{weight of NPs}} \quad (5)$$

2.6. Cell culture

B16 cell line, illustrative of mouse melanoma, was bought from the American Cell Culture Collection (ATCC). Cells were grown-up in Dulbecco's Modified Eagle Medium added with 10% fetal bovine serum (FBS), penicillin/ streptomycin (100 U/mL) and fungizone. Furthermore, the cells were maintained at the temperature of 370C, 95 percent air humidity and 5 percent CO2.

2.7. In vitro cell viability assay

The assessment of cell viability was performed as described by means of B16 melanoma cells. Momentarily, 6.5103 cells were seeded in 96 well-plates and upheld under optimum culture conditions till reaching 70% convergence. After this period, the cells were treated with dissimilar concentrations of nanoparticles (10, 50, 100 and 500 μg/mL) formerly filtered (0.45 μm filter) in their different NP, HNP and HNP-VD formulations. Cells were upheld 24 h after treatment and later they were submitted to cell viability examination. Firstly, cells were wash away with PBS and incubated with a solution of 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, Inc.) (5 mg/mL) diluted in DMEM 10% of FBS. The crystals of formazam, a product of the mitochondrial redox reaction of viable cells, were dissolved in 100 μL of dimethyl-sulfoxide (DMSO), and the staining intensity was determined by the Spectra-Max M2e-Molecular Devices equipment at wavelength 570 nm. The absorbance was directly proportional to the amount of living cells with active mitochondria. The results were expressed as % in relation to the control without treatment.

2.8. Cellular uptake

To assess the cellular uptake of the nanoparticles, B16 melanoma cells were treated with the dissimilar formulations containing the fluorescent marker Nile-red. Cells (2.010^4) were seeded in 24 well-plates and incubated under perfect conditions of cultivation for 24 hours. After, cells were treated for 3 hours with NP, HNP and HNP-VD, containing NR, at the concentrations of 50 and 100 μg/ml. Then, the culture medium was detached, the cells were washed 3 times with PBS and analysed through inverted fluorescence microscopy (Olympus IX71VR).

2.9. Statistical analysis

For the evaluation of the results, the ANOVA one-way test was used, followed by Tukey post-hoc test. The results were expressed as mean ± standard error. The GraphPad Prism 5.0VR program was used to evaluate the results and to generate the graphs. $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Synthesis of NP, HNP and HNP-VD

PLGA nanoparticles (NPs) were manufactured by the simple emulsification or solvent evaporation method. This practise is extensively reported in the literature for the making of polymeric NPs for various applications. The NPs were formed with properties such as size, zeta potential and PDI (polydispersity index) appropriate for application in drug delivery systems (Table 2). Among these properties, the small PDI was detected, confirming the homogeneousness of the sample magnitude distribution. From the images obtained in the FEG-SEM (Figure 2) it is probable to confirm the round morphology of the NPs as well as to check the size and the narrow distribution data detected via DLS.

As expected, rise in the size of the NPs occurred after addition a lipid layer on its surface. The PDI also amplified significantly. This may be due to the excess number of phospholipids resulting in the creation of micelles with dissimilar sizes and without a polymeric core in the stage of combination of these particles. Therefore, the use of 1:1 mass ratio (lipids: NPs) may lead to a difficulty in the manufacturing step due to the possible concurrent formation of lipid micelles. The zeta potential did not display any important rise in its value after the introduction of the lipid layer. The negative charges are a result of the presence of terminal carboxyl groups in the polymer. According to [22], the lipid-to-polymer ratio used in the combination of HNPs should be optimized since, when in surplus, lipids may synthesize micelles and liposomes absent of the polymeric core, which is reliable with our results. This fact can lead to accumulation and loss of active ingredients during the purification procedure.

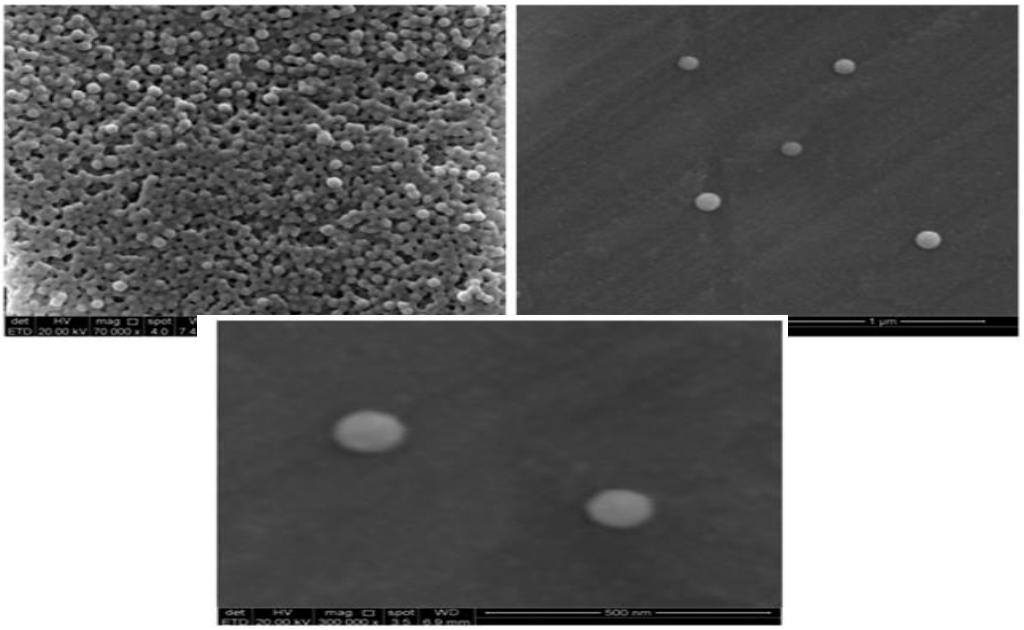

Figure 2. FEG SEM images from synthesized nanoparticles at 70,000x, 120,000x and 300,000x

Table 2. Characterization of NPs and HNPs by utilizing 1:1 lipid: NPs mass ratio.

Sample	Diameter (nm)	PdI	Zeta potential (mV)
NPs	133.6 ± 0.3	0.058 ± 0.012	−14.30 ± 0.70
NPHs	256.8 ± 38.4	0.286 ± 0.100	−13.59 ± 3.15

3.2. Optimization of lipid-to-polymer ratio

To evade the development of micelles without polymeric core and the extreme use of lipids to cover the HNPs, the coverage % of the lipid layer on the NPs was enumerated and the mass ratio (lipid: NPs) utilized in the synthesis was optimized using the following mass ratios: 1:10, 1:20 and 1:25 (w/w).

The HNPs were categorised as described formerly for the NPs and the results obtained are summarized in Table 3. As the ratio of lipid: NP declines, the particle size decreases, demonstrating that the lipid layer is less dense when less lipid is used to form the HNPs. The PDI was lower for particles formed with less lipid (1:10, 1:20 and 1:25) than those shaped with the highest ratio (1:1). The lower PDI shows that fewer, if any, lipid micelles may be forming. No noteworthy change in zeta potential was detected when reducing the lipids: NPs mass ratio.

Table 3. Characterization of HNPs formed with varying lipid: NPs mass ratio.

Lipid:NPs	Diameter (nm)	PdI	Zeta potential(mV)
1:1	256.77 ± 38.40	0.286 ± 0.10	−13.59 ± 3.15
1:10	209.80 ± 0.001	0.190 ± 0.19	−15.0 ± 1.56
1:20	189.30 ± 2.69	0.222 ± 0.08	−11.4 ± 2.19
1:25	163.20 ± 8.23	0.183 ± 0.01	−14.0 ± 1.30

The HNP coating % calculations can be achieved from the colorimetric method presented by Stewart together with H-NMR analysis, as presented [23]. The results of the H-NMR analysis are accessible in Figure 3, where the spectra provide the value of the proton integration of lactate, glycolate and ethylene oxide units. By calculating the ratio between these peaks, it is likely to quantify the % of DSPE-PEG2000 in the sample. Analysing the results, the ratios attained were 1.93:1, 6.92:1, 10.9:1 and 12.7:1 for the mass ratios of 1: 1, 1:10, 1: 20 and 1:25, correspondingly. The values attained for each compound relative to surface lipid density, molecules per NP and coating % are shown in Table 4. The total coverage % is given by the sum of the partial coverages of HSPC and DSPE-PEG2000. The total value for each mass ratios used in the HNPs preparations is shown in Figure 4.

As exposed in Figure 4, the mass ratio of 1:25 displays a NP coating of 97% and, therefore, it was determined that this ratio is optimal for the manufacture of HNPs and HNP-VDs. This condition is like to the work presented by Desai, where the authors used a 1:15 ratio, and where a ratio ranging from 10 to 20% was measured optimal. It is important to note that, in their work, the nano-precipitation method for the synthesis of the hybrid NPs was utilized but the polymer was not PLGA. Those who achieved the HNP preparation in two stages utilized the ratio of 1:1, but do not state the % of area covered by the phospholipid layer.

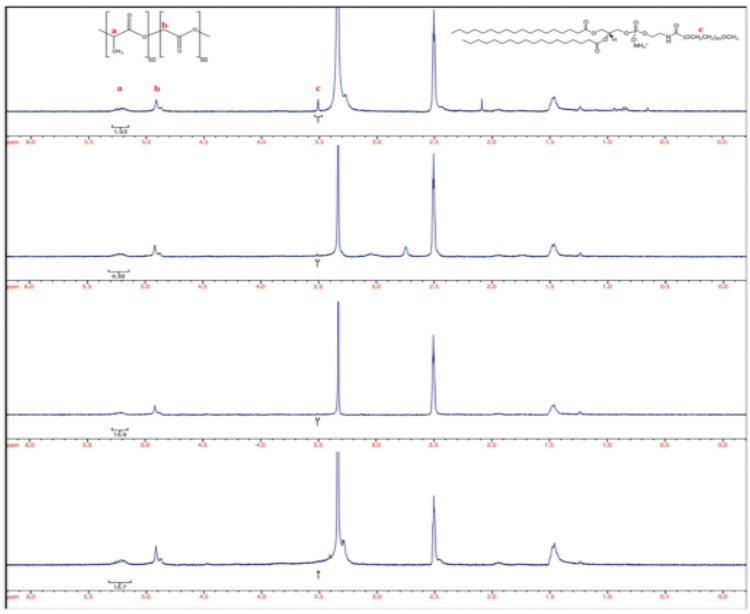

Figure 3. H-NMR spectra of hybrid nanoparticles synthesized. from top to bottom lipid; NPs mass ratio of 1:1, 1:10, 1:20 and 1:25.

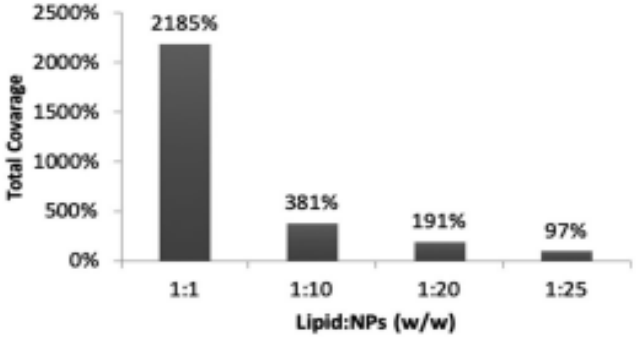

Figure 4. Total coverage of nanoparticles by utilizing various lipids: NPs ratios.

Table 4. Lipid quantification on surface of HNPs.

	HSPC			$DSPE\text{-}PEG_{2000}$		
Lipid:NPs (w/w)	Surface density (nmol mg^{-1} NP)	Molecules per NP (10^4)	Coverage (%)	Surface density (nmol mg^{-1} NP)	Molecules per NP (10^4)	Coverage (%)
1:1	544.8 ± 15.0	554.1 ± 15.2	1394 ± 38	172.9	175.8	791
1:10	106.1 ± 3.1	50.2 ± 1.4	210 ± 6	48.3	22.9	171
1:20	62.7 ± 3.5	16.4 ± 0.9	102 ± 6	30.6	8.0	89
1:25	24.4 ± 1.6	3.8 ± 0.3	33 ± 2	26.2	4.1	64

All of the HNPs and HNP-VDs used then in this work for drug release studies, In vitro cell viability and cellular uptake valuation were produced according to the improved fabrication conditions (1:25 lipid: NPs mass ratio). The results obtained from the description of the nano-carriers manufactured with the optimal conditions are described in Table 5. As expected, the diameter of the NPs rises when the phospholipid layer is introduced. Likewise, the HNPs that contain the vitamin D3 ligand (HNP-VDs) are to some extent larger than those without the ligand. However, zeta potential is unpretentious by the addition of the ligand. Figure 5 shows the images obtained by FEG-SEM of the samples HNP and HNP-VD.

Table 5. NPs, HNPs and HNP-VDs formed with optimal conditions.

	Diameter (nm)	PDI	Zeta potential (mV)
NPs	133.6 ± 0.3	0.058 ± 0.012	−14.3 ± 0.7
NPHs	163.2 ± 6.7	0.184 ± 0.011	−14.0 ± 1.1
NPHs-F	175.4 ± 6.5	0.199 ± 0.011	−13.9 ± 0.1

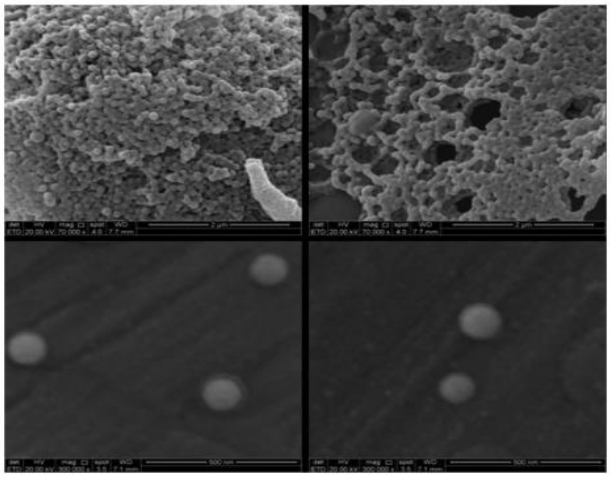

Figure 5. FEG SEM images from hybrid nanoparticles (left) and hybrid nanoparticles-vitamin D3 (right) synthesized with optimized lipid: NP ratio (1:25)

3.3. Drug release study

To perform release kinetics studies, NPs, HNPs and HNP-VDs comprising fluorescein were fabricated and the release studies were conducted over 6 days. The amount of fluorophore encapsulated within individual nanocarrier was enumerated and the results are shown in Table 6. In the first 24 h of incubation, 75, 62, and 57% of the fluorescein in the NPs, HNP, and HNP-VD were released correspondingly, configuring a burst release. For the consequent period, a slower release was detected where the cumulative release of fluorescein from the NPs was 88%, 75% for the HNP and 68% for the HNP-VD (Figure 6). The release behaviour is alike to the work presented where the total % released from the NPs was the uppermost followed by HNP and HNP-VD. It is hypothesized that this occurs because introducing the lipid layer rises the diffusion distance of the fluorescein, as does adding the targeting ligand (according to nanoparticle diameters in Table 6). The lipid layer may also affect division of the fluorescein within the nanoparticle, further hindering its release.

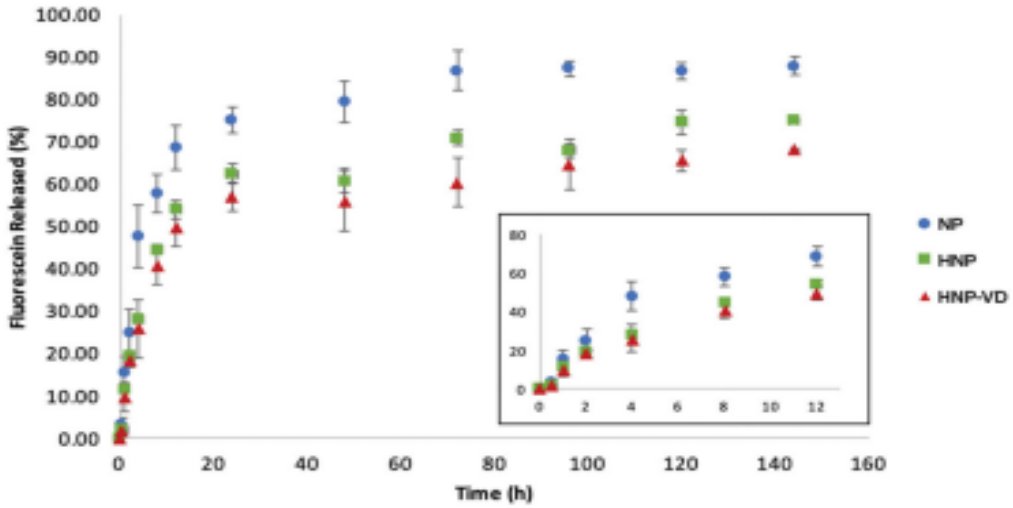

Figure 6. Fluorescein release behavior in PBS at pH 7.4; 0.01 M; 320C for the various samples.

3.4. In vitro cell viability

To validate the effect of the nanoparticles on cell viability, the diverse formulations (NP, HNP, and HNP-VD) were incubated with the B16 melanoma line [24], and the MTT assay was accomplished after 24 hours. After this period, the viability protocol was accomplished as described in the methodology, and results were expressed as a % relative to the untreated control. As depicted in Figure 7, cell viability was not changed with all the formulations tested, although it was detected a reduction of viable cells at the highest nanoparticle concentration (500 μg/mL) for HNP and HNP-VD, but the results were not statistically noteworthy. Notably, cells incubated with the HNP-VD formulation at 50 mg/mL exhibited a noteworthy rise in viability after 24 hours, demonstrating cell proliferation. Preceding study has shown that the activation of VD-VDR can encourage a proliferation stimulus to melanoma cells. The results attained here showed HNP-VD formulation as an important drug delivery system in melanoma cells.

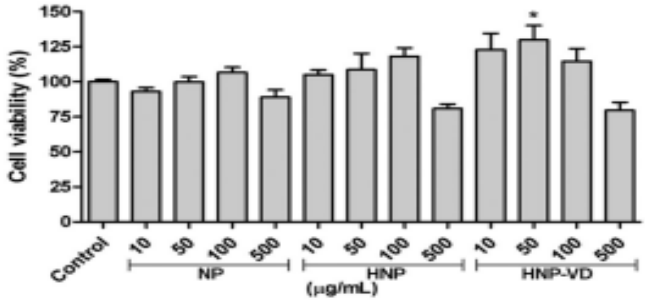

Figure 7. Evaluation of the cellular sustainability of B16 cells after treating with NP, HNP and HNP-VD preparations at conc. of 10, 50, 100 and 500 μg/ml.

Cells were upheld under optimal culture conditions for 24 hours under treatment. After this period, the viability protocol was achieved as described in the methodology. The results are expressed as a % relative to the untreated control. The experiments were performed 3 times in triplicate.

3.5. Cellular uptake study

Nanoparticle uptake by melanoma cells was assessed using fluorescence microscopy. After 3 hours of incubation with various formulations, it was detected that there was a alteration in the cellular distribution of the nanoparticles according to their specific formulation (Figure 8). NP (A) and HNP (B) HNP-VD (C) had more important localization in the cytoplasmatic and perinuclear domains, as indicated in Figure 8. After the incubation with the various nanoparticle preparations (NP, HNP and HNP-VD) at concentrations of 10 and 100 μg/mL, the cells were kept under optimal culture conditions for 3 hours and the fluorescence amount was assessed by an

inverted microscope. The images were taken from an objective with a 40x magnification. To facilitate the image of the proximity of HNP-VD to the nucleus of B16 melanoma cells, Figure 9 indicates the images obtained by brightfield and fluorescence microscopy, and the merged image utilizing a higher magnification power (400x). The images taken here clearly demonstrated that the HNP-VD nanoparticles were localized close to the nucleus of melanoma cells. This suggests that HNP-VD nanoparticles may be well-suited to provide therapeutics to the cell nucleus. Other studies have indicated the benefit of the internalization of NPs by tumour cells. It showed that the uptake of Nile-Red labelled NP by breast were placed nearer to the membrane of cells with decreased cytoplasmic distribution. On the other hand, cancer MCF-7 cells better the intracellular delivery of the encapsulated drug.

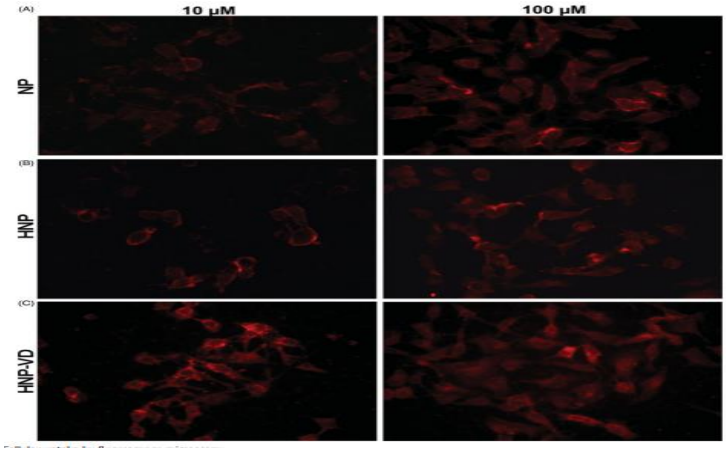

Figure 8. Cellular uptake by FM (fluorescence microscopy).

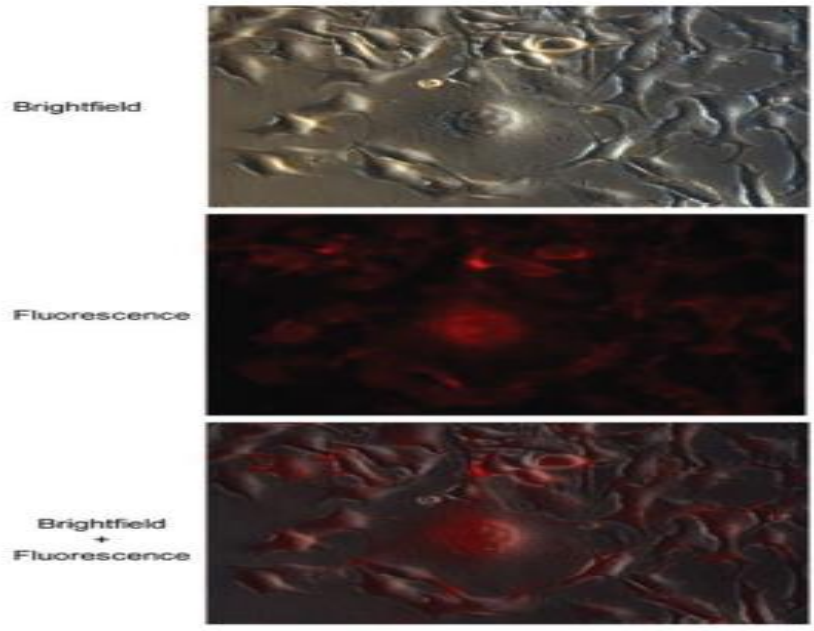

Figure 9. Cellular uptake and localization of HNP-VDs at the conc. of 100 µg/ml. The images were taken from an objective with magnification power of 400x.

4. Conclusion

This work presents the production of lipid nanoparticle hybrids, with (HNP-VDs) and without (HNPs) a pointing receptor in the surface. The mass ratio of lipid: NP was improved to obtain the optimum amount of lipids on the surface of the nanoparticles. According to the colorimetric process and the H-NMR study, HNPs were produced with 97 percent of their surface area covered by the lipid layer. In vitro release of fluorescein demonstrated an initial burst release of 75, 62, and 57 percent during the first 24 hours for NPs, HNP, and HNP-VD, respectively, followed by a gentler release for the left time. These results suggest that the nano-carriers used in this work can be used as regulated drug delivery systems for substances containing physio-chemical properties like fluorescein. Moreover, we can assume that the NPs and HNP did not promote considerable effects on melanoma B16 cells, though, HNP-VD lead to an increase of cell proliferation, probably due the communication VD-VDR. Cellular uptake data suggested that HNP-VD was concentrated in the perinuclear region of B16 melanoma cells, apparently due to the presence of the ligand vitamin D which affects nuclear receptor VDR. These results prove that HNP-VD is a good candidate for the development of affected melanoma treatment protocols as well as the specific transfer of encapsulated therapeutic agents to other cells containing nuclear VDR (vitamin D receptors).

5. Acknowledgement

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6. Conflict of interest

There is no conflict to declare.

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