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RESEARCH PAPER

Nano-encapsulation of Vitamin D_3 Active Metabolites for Application in Chemotherapy: Formulation Study and *in Vitro* Evaluation

Eyad Almouazen • Sandrine Bourgeois • Lars Petter Jordheim • Hatem Fessi • Stephanie Briançon

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ABSTRACT

Purpose Calcitriol (1,25-dihydroxyvitamin D_3), the active metabolite of vitamin D_3, is a potential anticancer agent but with high risk of hypercalcemia which limits the achievement of effective serum concentrations. Thus, calcitriol targeting delivery by nanoparticles may present a good solution.

Methods Vitamin D_3 active metabolites were encapsulated into polymeric nanoparticles and different formulation parameters were tested. The growth inhibitory efficiency of these nanoparticles was carried out *in vitro* on human breast adenocarinoma cells (MCF-7).

Results Using cholecalciferol (the inactive metabolite), different polymer and oil ratios were compared to select nanoparticles presenting high encapsulation efficiency and sustained release profile. Calcidiol/calcitriol loaded nanoparticles had good encapsulation efficiencies (around 90%) associated with sustained releases over 7 days and enhanced stability. Moreover, loaded nanoparticles showed similar growth inhibition to non-encapsulated metabolites of vitamin D_3 on day 4 and higher activities on days 7 and 10 after treatment initiation.

Conclusion The nano-encapsulation of vitamin D_3 active metabolites may offer a new and potentially effective strategy for vitamin D_3-based chemotherapy overcoming its actual limitations. The targeting delivery of vitamin D_3 metabolites should be encouraged.

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INTRODUCTION

Vitamin D_3 (cholecalciferol) is a steroid hormone produced in the skin or ingested from dietary sources. The metabolism of Vitamin D_3 into its active form begins in the liver where it is hydroxylated to 25-hydroxyvitamin D_3 (calcidiol) followed by a second hydroxylation in the kidney into 1,25-dihydroxyvitamin D_3 (calcitriol) (1). The classical role of calcitriol is calcium and phosphate blood level maintenance in coordination with parathyroid hormone. Calcitriol has also immune modulating potential and regulatory effects on the proliferation and differentiation of benign and malignant tissue (2). *In vitro* and *in vivo* studies have shown the antineoplastic activity of calcitriol in various types of malignancy including carcinoma of the bladder, breast, prostate, lung, pancreas, bone and colon, glioma, neuroblastoma, melanoma and leukemia (3). The mechanisms of calcitriol's antineoplastic activity could differ between tumor models and experimental conditions, and more investigations are needed to clearly understand these mechanims (3). Calcitriol has anti-tumoral activity in supraphysiological dose (10^{-9} – 10^{-6}M *in vitro* and $>10^{-9}$ M *in vivo*) associated with a high risk of hypercalcemia. The early clinical studies to evaluate the anti-cancer activities of calcitriol showed the interest of calcitriol as chemotherapeutic agent but highlighted the combined risk of unmanageable hypercalcemia (4). Recent clinical trials showed that an intermittent (weekly) high dose of calcitriol could limit the hypercalcemia and the combination of calcitriol with other chemotherapeutic agents could be useful (5). Two pharmaceutical formulations (oral and intravenous) of calcitriol are available but they are not suitable for anticancer treatment due to the difficulty to maintain active systemic level of

calcitriol (6). The last published clinical trial (ASCENT-2) using an improved oral formulation (DN-101, Novacea Pharmaceuticals) showed inferior survival in the calcitriol arm which could be explained by more side effects and the fact that the maximal tolerated dose of calcitriol was not used (7). Thus, calcitriol treatment strategies still need more investigations.

25-hydroxyvitamin D_3 (calcidiol) is the major circulating metabolite of vitamin D_3. Its serum concentration is 1000 fold higher than that of calcitriol but it has 500 fold lower affinity for vitamin D receptor (VDR) than calcitriol (8). It can be converted into calcitriol by 1α-hydroxylase (encoded by the gene CYP27B1) classically located in the kidney. The expression of CYP27B1 in extra-renal sites such as brain, colon, skin, macrophages and other has been documented indicating localized synthesis of calcitriol (9). The presence of 1α-hydroxylase is also documented in cancer tissues like breast and prostate cancer, glioma and other with different intensities (9). Barreto *et al.* reported the antiproliferative effects of calcidiol on human prostatic epithelial cells that express 1α-hydroxylase at 100 nM (10). Besides, Lou *et al.* showed the direct gene regulatory properties of calcidiol on the CYP27B1 knockout cells and its direct antiproliferative effects on human LNCaP prostate cancer cells at concentration 50 fold higher than the effective concentration of calcitriol (11). Moreover, it was shown that solid tumors like glioma, lung, prostate and breast cancer are infiltrated by immune cells such as tumor-associated macrophages (TAM) (12). The expression of 1α-hydroxylase and the conversion of calcidiol into calcitriol are well documented in macrophages (13). In TAM, the activity of 1α-hydroxylase has not been widely investigated, but Yokomura *et al.* observed an increase of 1α-hydroxylase expression in lung cancer-infiltrated macrophages (14). Therefore, it can be postulated that non-1α-hydroxylated analogs like 25-hydroxyvitamin D_3 could have a potential anti-cancer activity which increases the local drug concentration without systemic side effects.

From all above, one can suppose that the development of calcitriol treatment should be focused on targeting cancer cells and sustaining effective concentration in tumors environment. Calcitriol vectorisation may ensure specific action on cancer cells and increase the concentration delivered while avoiding calcitriol-related side effects and permitting combination therapy. For this purpose, biodegradable polymeric nanoparticles (NP) are possible carriers of vitamin D_3 active metabolites. NP can modify release and distribution profiles of drugs allowing effective delivery to be improved and toxic effects to be lowered (15). It was well documented that NP could be internalized by targeted cells increasing drug intracellular delivery and its therapeutic effects *via* enhanced intracellular stability and sustained release (16). Moreover, polymeric nanoparticles could offer a selective drug delivery to tumor tissue either by passive targeting with the enhanced permeability and retention effect (EPR) or by active targeting using functionalized NP (17).

The nano- and micro-encapsulation of vitamin D_3 towards a therapeutic use has been poorly reported. Some papers deal with the micro-encapsulation of cholecalciferol as a drug model or as a nutrient supplementation (18). Luca *et al.* encapsulated cholecalciferol into cellulose acetate microspheres as anti-oxidizing agent to enhance pancreatic islet cell viability and function (19). Nguyen *et al.* presented the formulation of calcitriol-loaded microparticles using poly(vinyl neodecanoate-crosslinked-ethyleneglycol dimethacrylate) as polymer to ensure extended anti-cancer concentration after local hepatic injection (20). Moreover, cholecalciferol was also encapsulated into hydrophobic alginate nanoparticles for sustained release oral form (21,22). However, to the best of our knowledge, the nano-encapsulation of calcitriol or other active analogues toward cancer treatment has not yet been reported.

The aim of the present study was to develop a new formulation of vitamin D_3 suitable for anticancer application using polymeric nanoparticles as drug carriers, and to evaluate the *in vitro* antiproliferative activity of such preparations. Poly(D, L)lactic acid (PLA) is a non-toxic biodegradable polyester, was chosen to prepare NP by nanoprecipitation method. Due to the cost of calcidiol and calcitriol, the inactive form of vitamin D_3 (cholecalciferol) was used in the optimization step. The adapted NP formulation was selected with regard to size, encapsulation efficacy and release profile of the drug. NP mean size near 200 nm is preferred in order to be sterilized by filtration. Reduced burst release is also important to minimize the undesired drug release before NP reach tumor cells. The selected nanoparticle formulation was then loaded with calcitriol or calcidiol to assess its potential growth inhibitory efficacy in comparison with non-encapsulated form.

MATERIALS AND METHODS

Materials

The nanoparticle polymer used was poly$_{(D,L)}$lactic acid (PLA, MW 20000 g/mol, Evonik Birmingham Laboratories, USA). The studied vitamin D_3 metabolites were cholecalciferol (Vitamin D_3, Sigma-Aldrich, France), calcidiol (25-hydroxyvitamin D_3, Tocris bioscience, UK) and calcitriol (1.25-dihydroxyvitamin D_3, Cayman chemicals, USA). The fluorescent molecule, Nile red (NR), was purchased from Sigma-Aldrich, France. Montanox® VG 80 and Miglyol®829 (caprylic/capric/succinic triglyceride) were purchased respectively from Seppic and Sasol, France. Human breast adenocarcinoma cells (MCF-7) were obtained from American Type Culture Collection, USA.

Polymeric NP Preparation

NP were prepared by using the nanoprecipitation technique (23). First, polymer (poly(D,L)lactic acid) and organic oil (Miglyol®829) were dissolved in acetone at the concentrations listed in Table I. Ethanolic solutions of the cholecalciferol, calcidiol or calcitriol were prepared and then added to the previous acetone solution to achieve a final concentration of drug about 0.025% (*w*/*w*). This organic phase was then poured into the aqueous phase which contains 0.05% of Montanox® VG 80, a non-ionic surfactant used to stabilize NP formulation. NP were instantaneously formed by the rapid solvent diffusion inducing polymer precipitation. The NP suspension was stirred moderately at room temperature for 10 min. Acetone and a part of the water were removed under vacuum using a rotary evaporator (Rotavapor® RE-140, Büchi, Switzerland). Formulation variables of cholecalciferol-NP are listed in Table I. Similar formulas were prepared as controls (blank-NP) using ethanolic solution containing no drug. Fluorescence labeled NP were prepared in the same way by incorporating Nile red (NR) in the organic solution at a concentration of 0.0125% (*w*/*w*). The nanoparticle suspensions were stored at 4°C in hermetically closed vials. Before the *in vitro* application, NP suspensions were sterilized by filtration through 0.2 µm syringe filter (Minisart®, Sartorius, France). All formulations were made in triplicate.

Characterization of Nanoparticles

The particle size distribution was measured using photon correlation spectroscopy (Malvern Zetasizer® nano-serie) with NP suspension diluted in deionized water at room temperature. The zeta potential of NP was also investigated using a Zetasizer® (Nano-serie, Malvern). Results were expressed as the average of three measurements. The size distribution is given by polydispersity index (PdI).

The NP were also characterized by their encapsulation efficiency. The total drug content in NP suspension was determined by dissolving 100 µl of NP suspension in acetonitrile to form a solution which was then filtered through a 0.45 µm syringe filter (Minisart® NY15, Sartorius, France) and analyzed by RP-HPLC methods as described below. Non-encapsulated drug (free) was determined in the supernatant after ultracentrifugation at 18,000 g for 30 min of the NP suspension. Encapsulation efficiency (%) was calculated by the difference between the total and free drug concentrations divided by the theoretical-calculated total drug concentration. The drug content in sterilized NP suspension was also determined.

Table I NP Formulation Variables: Percentages of Polymer (PLA) and Oil (Miglyol® 829) Dissolved in the Organic Phase during Cholecalciferol-NP Preparation

Sample	Polymer % (*w*/*w*)	Oil % (*w*/*w*)	Polymer : oil ratio
NP1	0.5	0	1:0
NP2	0.5	0.5	1:1
NP3	0.5	1	1:2
NP4	0.5	2	1:4
NP5	1	0.5	2:1
NP6	1.5	0.5	3:1

In Vitro Release Study

In vitro release of drug from NP was conducted by a centrifugation method at 37°C for 7 days. The release medium was Phosphate Buffer Saline (PBS 0.01 M, pH 7.4) containing 0.01% of Sodium Dodecyl Sulfate (SDS) to ensure sink conditions. NP suspension was diluted in 50 ml of release buffer (final concentration was 4 µg/ml of drug) and placed under moderate magnetic stirring (100 rpm) at 37°C. At predetermined time intervals, samples of 1 ml were withdrawn, centrifuged for 30 min and the drug released from NP was quantified in the supernatant by RP-HPLC. Three experiments were conducted with three different NP preparations. The selected formulation was also examined with daily buffer change to overcome the incomplete release. In this case, the release medium containing NP was centrifuged daily and a fresh release medium was added to resuspend the NP.

Determination of the Stability of the Encapsulated Drug

To study the *in vitro* stability of encapsulated drug in cell culture conditions, NP were suspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, France) supplemented with penicillin-streptomycin (Invitrogen, France) in order to obtain a final drug concentration of 4 µg/ml. Free drug was used as control at the same concentration. All samples were incubated at 37°C for 7 days. Each day, samples of 0.5 ml were taken and analyzed by RP-HPLC to determine the residual drug amount. The study was performed in triplicate.

Partition of Cholecalciferol Between oil and Polymer

In order to estimate the distribution of encapsulated cholecalciferol inside nanoparticles, cholecalciferol partition between oil and polymer was conducted. For this purpose highly concentrated acetone solutions of drug, polymer (PLA) and oil (Miglyol® 829) were prepared in triplicate with the same polymer:oil ratios used for NP preparation. After vigorous agitation these solutions were placed into centrifugal evaporator (miVac, Genevac, UK) which allows evaporating the

acetone and separate polymer and oil in two phases. Samples of the oil phase were collected and analyzed by RP-HPLC in order to quantify the amount of cholecalciferol contained in the oil. Results are expressed as both the concentration and percentage of cholecalciferol in the oil phase as a function of the polymer:oil ratio.

RP-HPLC Methods

Cholecalciferol was analyzed by RP-HPLC Waters 600 Controller and Waters 717 Auto sampler (Waters, France) on a X-Terra MS C18 column (5 μm, 4.6×250 mm). An isocratic mobile phase consisting of 10% of acetonitrile, 85% of methanol and 5% of 0.5% acetic acid solution was used with a flow rate of 1.3 ml/min. The photodiode-array detector (Waters 2996) was adjusted at λ =265 nm. A 20 μl aliquot sample was injected. The calibration curve for the quantification of cholecalciferol was linear over the range of concentrations used, from 0.1 to 5 μg/ml (r^2=0.999).

The quantification of calcidiol was also performed under the same conditions with a flow rate of 1 ml/min and a calibration curve between 0.1 and 5 μg/ml. Using the same column, calcitriol was analyzed with 1 ml/min flow rate of an isocratic mobile phase consisting of 10% of acetonitrile, 80% of methanol and 10% of 0.5% acetic acid solution.

Cell Culture

MCF-7 breast cancer cells were cultured in T75 flask with Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, France) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS). When the cells reach 80% of confluence, they were washed by PBS, trypsinized and subcultured.

In Vitro Evaluation of the Growth Inhibitory Activities of Calcidiol/Calcitriol Loaded NP

MCF-7 cells were used to assess the anti-proliferation activities of encapsulated calcidiol or calcitriol. Briefly, cells were seeded in 96-well plates (1000 cells/well) and cultured for 24 h. Cells were then exposed to either calcidiol/calcitriol loaded NP, or ethanolic solution of calcidiol/calcitriol at the desired concentrations (10^{-8} – 10^{-6}M). Cells exposed to unloaded NP (blank-NP) and ethanol (0.1%) were used as negative controls for loaded NP and free form, respectively. After 4 days, cells were washed and fresh completed medium was added. The medium was changed every 3 days. At 4, 7 and 10 days after the initiation of treatment, cell proliferation was determined using the MTT assay as described earlier by Jordheim *et al.* (24). Three individual experiments were done for each time point. The results are presented as a percentage of cell density compared to the corresponding control.

In Vitro Evaluation of Intracellular Delivery Using NR-Labeled NP

MCF-7 cells were seeded in 6-well plate containing cover slips at a density of 20,000 cells per well for 24 h. Cells were then exposed to Nile red-labeled NP and free Nile red (dissolved in ethanol) at a final concentration corresponding to 0.4 μg/ml of NR. At 4 days, 7 days and 10 days after the initiation of incubation, cells were washed with PBS and fixed with paraformaldehyde 4% (Invitrogen, France) for 10 min at room temperature. Cells were then rinsed with PBS and mounted with one drop of Vectashield (Vector Laboratories). Fluorescence microscopy (Leica DMI3000) was used to evaluate the intracellular location of NP.

RESULTS

Study of Formulation Parameters on the Encapsulation of Cholecalciferol

In the aim of developing calcidiol and calcitriol NP, we first determined the optimized formulation parameters. Cholecalciferol, the inactive form of vitamin D_3, was employed in this step. Six NP formulations were prepared in triplicate using poly(D,L)lactic acid and Miglyol®829 with different polymer:oil ratios as presented in Table I including one formulation of nanospheres (NP1) and five nanocapsules (NP 2–6). These NP were characterized by their size distribution, their encapsulation efficiency and their release profile as presented in Table II and Fig. 1. The six NP had different mean sizes varying between 170 and 280 nm and different polydispersity indexes less or equal to 0.2. The release profile of cholecalciferol from these NP can be divided into three phases: a burst release in the first hour, a sustained release over the next 23 h and a slow release between 24 h and 168 h (Fig. 1).

Initial comparison between nanospheres (NP1) and nanocapsules (NP2) showed that NP1 had the smallest particle mean size (168±11 nm) and low encapsulation efficiency (69.8±2.4%) associated with high burst release (60% in the

Table II Effects of Formulation Variables on Cholecalciferol-NP Properties

Sample	Particle mean size (nm)	Polydispersity index	Encapsulation efficiency %
NP1	168±11	0.07±0.02	69.8±2.4
NP2	188±8	0.06±0.01	91.8±1.5
NP3	222±7	0.09±0.03	94.3±2.0
NP4	283±23	0.20±0.05	97.2±0.6
NP5	230±24	0.12±0.09	90.6±2.4
NP6	253±17	0.14±0.01	92.5±1.3

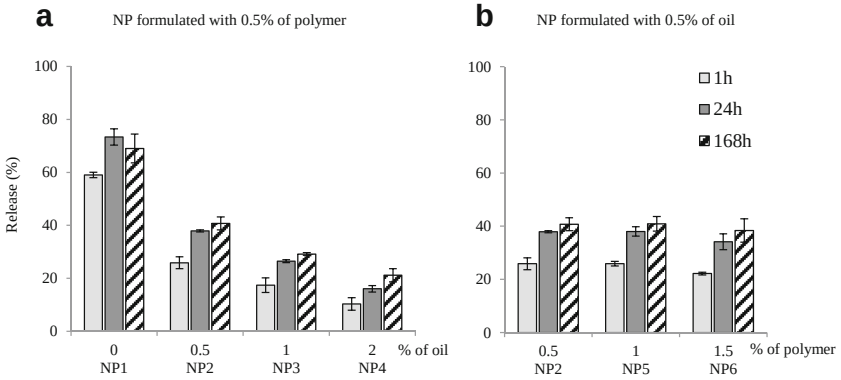

Fig. 1 Effects of formulation variables on *in vitro* cholecalciferol release profile assessed by centrifugation method under sink conditions. NP were dispersed in PBS containing 0.01% SDS at a final concentration of cholecalciferol of 4 μg/ml. Each bar presents the mean of released percentages obtained from three different preparations and error bars represent the standard deviations. (**a**) NP formulated with increased concentrations of oil in organic phase. (**b**) NP formulated with increased concentrations of polymer in organic phase.

first hour). In contrast, NP2 present good encapsulation efficiency about 92% and a sustained release profile with 25.9% at 1 h and up to 40.7% at 168 h. Thus, nanocapsules are more suitable for vitamin D_3 encapsulation than nanospheres.

Adding more oil in the NP formulation (NP3 and NP4) enhanced the encapsulated quantity up to 94.3% and 97.2%, for NP3 and NP4, respectively. This was associated with an increase in NP mean size. The release profile was also more sustained in comparison with NP2 as shown in Fig. 1a. The burst release decreased to 17.4% and 10.3% and the percentage released after 168 h also decreased to 29.2% and 21.2%, for NP3 and NP4, respectively. The role of oil phase in vitamin D_3 encapsulation was remarkably interesting.

Increasing the percentage of polymer (NP5 and NP6) augmented NP mean size while the encapsulation efficiency was slightly affected. The release profile of NP5 (2:1 polymer:oil ratio) did not change in comparison with NP2. In the case of polymer:oil ratio 3:1 (NP6) a small reduction of the burst release was observed (22.2% for NP6 compared to 25.9% for NP2) as shown in Fig. 1b. The formulation of NP with 4:1 polymer:oil ratio wasn't possible due to the precipitation during evaporating step. Therefore, increasing the percentage of polymer in nanoparticles formulation had no interest toward vitamin D_3 encapsulation.

To understand the advantageous role of the oil and the difference between these nanoparticles, cholecalciferol partition between oil and polymer was evaluated. Acetone solutions containing cholecalciferol, polymer and oil in the same ratio utilized in NP2-6 were prepared. After acetone evaporation, two separate phases were obtained; the upper phase contained oil and the lower phase consisted of polymer. Measurement of cholecalciferol content in the oily phase allowed us to calculate the percentage of cholecalciferol dissolved in oil and to estimate its partition between oil and polymer as presented in Table III. When equal quantities of oil and polymer were used, we observed 84.1±3.4% of cholecalciferol in oil. This result showed the hydrophobic properties of cholecalciferol and the oil tendency to entrap it. When more oil was added (polymer: oil ratio 1:2 and 1:4) this percentage was significantly increased up to 92.0±2.5% and 98.8±2.9% (P <0.05 and <0.01, respectively as compared to 1:1 ratio). In the case of ratios 2:1 and 3:1, concentration and percentage in oil had a small tendency to decrease, confirming the low effect of polymer on cholecalciferol encapsulation.

From all the results above, NP4 showed adequate characteristics for our therapeutic application except their size (mean size about 280 nm and PdI 0.2). Based on our laboratory experiences, the same composition of NP4 (polymer:oil ratio 1:4) was formulated in diluted concentration using polymer at 0.25% and oil at 1% (w/w) in the organic phase. The obtained NP (called cholecalciferol-NP) had a suitable size near to 200 nm and were used in subsequent experiments.

Preparation, *In Vitro* Release, and Stability of Selected NP

Based on the selected formulation, three NP were prepared and loaded with cholecalciferol (cholecalciferol-NP),

Table III Evaluation of Cholecalciferol Partition Between oil (Miglyol® 829) and Polymer (PLA) Presented by the Cholecalciferol Concentrations in oil Phase and the Percentage of Cholecalciferol Dissolved in Oil. The Values are Statistically Different (*: $P<0.05$, **: $P<0.01$, $n=3$) as Compared to NP2

Sample	Polymer:oil ratio	Concentration in oil (μg/mg)	Percentage in oil (%)
NP2	1:1	41.1±1.9	84.1±3.4
NP3	1:2	22.9±0.2	92.0±2.5*
NP4	1:4	12.2±0.5	98.8±2.9**
NP5	2:1	36.6±2.9	79.3±9.0
NP6	3:1	37.4±2.6	77.3±4.3

calcidiol (calcidiol-NP) and calcitriol (calcitriol-NP). These NP were characterized by their size distribution, their surface charges (zeta potential) and their encapsulation efficiency as shown in Table IV. A slight decrease in the encapsulation efficiency of cholecalciferol-NP compared to NP-4 was observed (91.9% *vs* 97.2%). Changing the encapsulated drug had no effect on NP size; these NP had a similar size distribution with a mean size near to 200 nm and PdI about 0.1. The encapsulation efficiency varied according to the encapsulated molecules due to the differences of their hydrophobicity. Calcitriol-NP had the lowest value (87.8±0.6%) as compared with the others.

The release profiles of these NP were evaluated by centrifugation method under sink conditions (data not shown). The release profiles reached a plateau after 24 h so the release study was carried out another time with daily buffer change to simulate infinite sink conditions and the obtained release profiles are presented in Fig. 2. Continuous release was observed over 168 h for the three NP indicating a sustained release profile and the ability to release the entire encapsulated drug. Calcidiol-NP released 27% in the first 24 h and the cumulative release reached 80% at 168 h. 64% of calcitriol was released in the first 24 h and 87% after 96 h. Calcitriol-NP showed a rapid release due to the high solubility of calcitriol in the release medium.

Figure 3 shows the *in vitro* stability of encapsulated drugs in cell culture medium which can partially represent the complex physiological conditions. Free or nano-encapsulated drugs were dispersed in DMEM medium and the drug amount was quantified by HPLC after 1, 3 and 7 days (168 h) of incubation. Cholecalciferol free form degraded rapidly since only 22.6% and 8.7% were detected after 24 h and 168 h respectively. In contrast, 41.4% of cholecalciferol remained after 168 h for the encapsulated form. Encapsulation also enhanced the stability of calcidiol with 51.9% remaining after 168 h while free form was totally degraded within the first 24 h. Moreover, NP protected calcitriol from degradation and conserved 79.8% after 24 h and 18.6% after 168 h. From results described in Figs. 2 and 3we can conclude that PLA based NP are suitable carriers for calcidiol and calcitriol presenting sustained release profiles and enhanced drug stability.

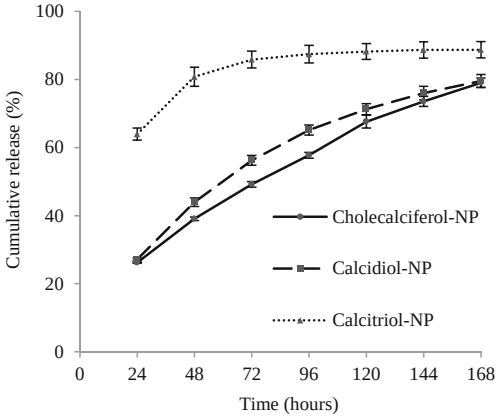

Fig. 2 *In vitro* release profile of nanoparticles conducted with daily buffer change. Results are mean values from three independent experiments and error bars represent the standard deviations.

Cell Growth Inhibition by Calcidiol/Calcitriol-Loaded NP

The growth inhibition activity on MCF-7 cells of encapsulated calcidiol and calcitriol was evaluated at three concentration (10^{-8}, 10^{-7} and 10^{-6}M) and compared to non-encapsulated forms. MTT assay was carried out on days 4, 7 and 10 after treatment initiation to evaluate cell survival in the different conditions. No significant changes in cell survival were observed on cells exposed to blank-NP or ethanol during the experiment (data not shown). Figure 4 showed similar effects for free and nano-encapsulated drugs were observed on day 4, whereas encapsulated drugs induced more potent inhibition of cell survival on days 7 and 10. In particular, nano-encapsulated calcidiol induced a statistically significant growth inhibition at 10^{-6}M as compared to free calcidiol ($p<0.05$).

On day 4, free and encapsulated calcidiol reduced the cell proliferation to 53% ($P<0.01$ compared to corresponding controls) only when high concentration was used (10^{-6}M). This effect was sustained for free calcidiol until 7 days while calcidiol-NP decreased cell proliferation to 31% on day 7 and still had effect after 10 days (65%, $P<0.05$). Moreover, calcitriol as expected presented higher effect than calcidiol. On day 4 free and encapsulated calcitriol showed anti-proliferative activities about 63% and 50% ($P<0.01$) when 10^{-7} and 10^{-6}M were used, respectively. Seven days after treatment initiation, 10^{-7}M of calcitriol maintained its activity for free and encapsulated forms while it had no more effect after

Table IV NP properties. The selected NP Formulation was Prepared in Diluted Conditions and used to Encapsulate Calcidiol and Calcitriol

Sample	Particle mean size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Cholecalciferol-NP	212±6	0.08±0.01	−31.3±2.1	91.9±0.7
Calcidiol-NP	202±14	0.09±0.02	−29.9±2.2	93.7±0.9
Calcitriol-NP	201±14	0.05±0.03	−29.8±2.5	87.8±0.6

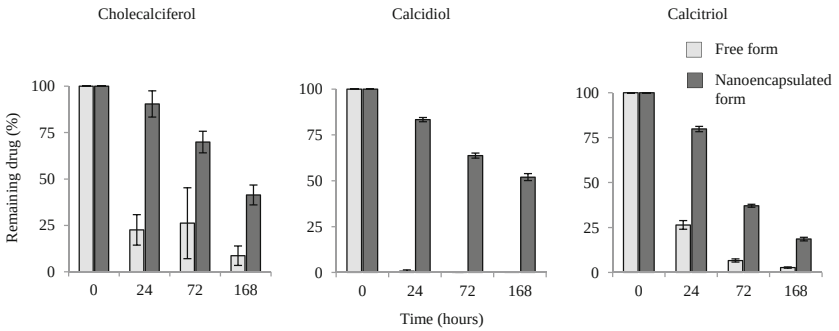

Fig. 3 Encapsulation of cholecalciferol, calcidiol and calcitriol enhanced their stabilities. Stability test carried out in cell culture conditions over 7 days. Data presented are for nano-encapsulated form and for free form. Results are mean values from 3 independent experiments and error bars represent standard deviations.

10 days. Incubation with 10^{-6}M of calcitriol suspends cell proliferation for 10 days with 28% cell survival for free calcitriol and 15% for calcitriol-NP. From all above, it was clear that nanoparticles could keep and sustain the anti-proliferative efficacy of calcidiol and calcitriol on MCF-7 cells *in vitro*.

In Vitro Evaluation of Intracellular Delivery by NP

To better understand the role of NP in enhancing growth inhibition efficiency, the internalization and the persistence of NP inside MCF-7 cells were investigated by fluorescent

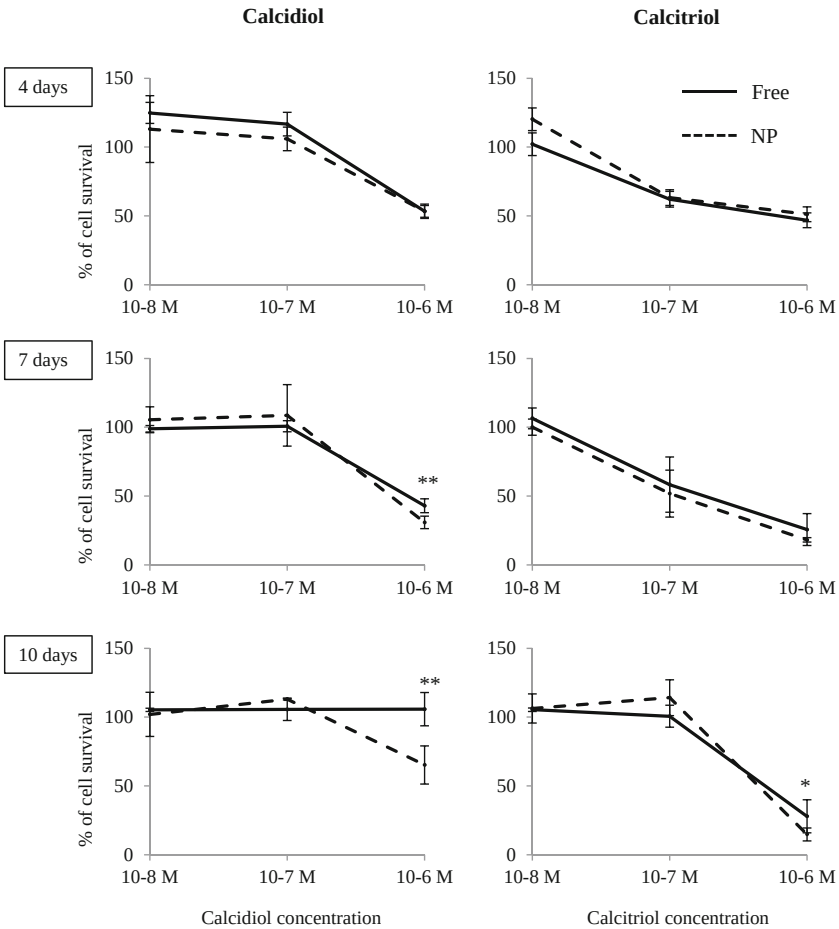

Fig. 4 Effects of calcidiol-NP and calcitriol-NP on MCF-7 cell proliferation assessed by MTT test. MCF-7 cells were plated in 96-well at cell density of 1000 cells/well and exposed to calcidiol-NP, calcitriol-NP or their ethanolic solutions at various concentrations (10^{-8}, 10^{-7} and 10^{-6}M). Blank-NP treated cells and ethanol (0.1%) treated cells were used as controls for encapsulated and free drugs, respectively. Cells densities are expressed as percentages of corresponding control. Values are the mean of three independent experiments and error bars represent standard deviations. The values are statistically different (*: $P<0.1$, **: $P<0.05$) when calcidiol/calcitriol-NP compared to free form.

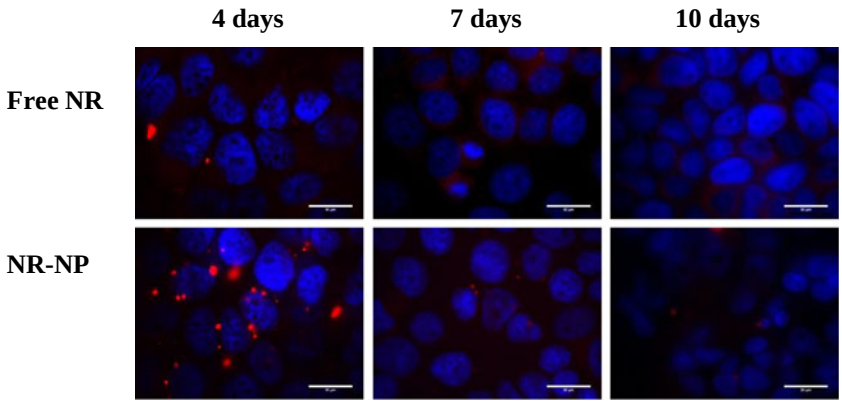

Fig. 5 Using Nile red (NR, fluorescent molecule) the enhancement of intracellular delivery by NP was evaluated. MCF-7 cells were exposed to free NR (ethanolic solution) or to NR-labeled NP. At days 4, 7 and 10, cells were observed by fluorescent microscopy. Cell nucleus is stained with DAPI (blue), and NR and NR-NP can be observed inside cells as a red fluorescence (bar=30 μm).

microscopy using Nile red (fluorescent molecule) to label NP. Four days after incubation, important fluorescent granular materials were detected inside cells treated with NR-labeled-NP (Fig. 5). Moreover, some fluorescent spots were detected inside cells at day 7 and 10. Conversely, when cells were exposed to non-encapsulated NR (ethanolic solution), only few fluorescent points were detected at day 4. The residual fluorescence detected after 7 and 10 days with free NR was less intense than with RN-NP. These results indicate that NP could enhance intracellular delivery of encapsulated drug since at day 4 remarkable differences were observed between encapsulated and free form of NR.

DISCUSSION

The anticancer effects of calcitriol, the active metabolite of vitamin D_3, have been well documented both *in vitro* and *in vivo*. However, using calcitriol in cancer treatment needs supraphysiological doses that are associated with side effects such as hypercalcemia. This limits the achievement of an effective serum concentration in clinical trials (3). Moreover, calcidiol (25-hydroxyvitamin D_3) presents also anticancer activities at a high dose and it has less calcemic effects (11). We evaluated the ability of polymeric NP to overcome these limitations and to present new formulations suitable for calcitriol-based chemotherapy that would allow sustaining high drug concentrations *in vivo*. Poly(D,L)lactic acid (PLA, Mw 20000) was chosen to prepare NP by nanoprecipitation method. It is a synthetic polyester polymer approved by the Food and Drug Administration (FDA) with unique properties of biocompatibility and biodegradability which hydrolyzes to nontoxic metabolites in physiological conditions (25).

In this study we focused on the effects of formulation parameters on the encapsulation of active metabolites of vitamin D_3 Nanoprecipitation technique was used to prepare NP because it is a simple and highly reproducible method allowing the production of small nanoparticles with narrow size distributions. Moreover, nanoprecipitation is a well-adapted technique for the encapsulation of hydrophobic molecules (23). Cholecalciferol was used in the optimization step and the obtained nanoparticles (NP 1–6) helped in the selection of adapted parameters. Nanocapsules (NP2) were more suitable than nanospheres (NP1) due to the presence of oily core that can dissolve hydrophobic cholecalciferol and enhance its entrapment as reported before for other similar drugs (26). To enhance the encapsulation efficiency and to obtain a longer release profile, different percentages of oil or polymer were tested (Table 1). These NP had different sizes, encapsulation efficiencies and release profiles that showed the advantageous role of oil and the little effect of polymer in enhancing encapsulation efficiency.

The release profiles of these NP were divided into three phases. Burst release in the first hour presented the release of surface-attached cholecalciferol. Sustained release over the following 23 h could be attributed to the diffusion of cholecalciferol from NP core into the release medium. After diffusion equilibrium between NP and release medium had occurred, slow phase release took place between 24 h and 168 h. This equilibrium was the result of the partition of cholecalciferol between the colloidal system and the external aqueous phase (release medium) as discussed before for the drug release from colloidal systems by Washington *et al.* (27). From this point of view, the differences in release profile between these formulations could be explained as follows. The decrease of the burst release observed by adding more oil reflects the reduction of cholecalciferol linked to the surface that could be explained as a consequence of increased the encapsulation efficiency and the amount of cholecalciferol entrapped in the nanoparticle core. Conversely, adding more polymer increases the thickness of NP layer without affecting the drug entrapment or its release from NP (Fig. 1B). The amount released during the next 23 h was also decreased for NP2, NP3 and NP4 (about 12%, 9% and 6% respectively,

Fig. 1) illustrating that the diffusion equilibrium was achieved with lower concentration of cholecalciferol in medium of release. Adding more oil decreased cholecalciferol concentration inside NP and therefore the partition of cholecalciferol between NP and the release medium is in favor of NP. Similar observations were reported by Calvo *et al.* for indomethacin (28) and by Teixeira *et al.* for 3-methoxyxanthone (26) where the release of the drug is depending on its partition between NP and release medium. These explanations are in accordance with our investigations of cholecalciferol distribution inside NP (Table 3). When polymer and oil were separated from acetone solution, about 84% of cholecalciferol was detected in oil phase and this percentage was increased with increasing oil quantity. Besides, cholecalciferol concentration in oily phase was diluted when more oil was added.

From this optimization step, NP4 formulation was selected to continue our study and it was prepared in diluted conditions to obtained suitable particle size around 200 nm. An increase of the burst release was observed for final cholecalciferol-NP formulation compared to NP4 due to the increase of specific surface of NP when their size decreased. These NP were then loaded by either calcidiol or calcitriol and variation in the encapsulation efficiency was observed. Based on the chemical structure, calcidiol has one hydroxyl group and calcitriol has two hydroxyl groups more than cholecalciferol, these drugs can be classified depending on their hydrophobicity as follow : cholecalciferol > calcidiol > calcitriol. Therefore, it was not surprising that calcitriol-NP had moderate encapsulation efficiency and rapid release profile as compared to the other ones. We also observed an inverse relationship between the hydrophobicity of the drug and its *in vitro* release. These nanoparticles showed sustained release profile (Fig. 2) and enhanced stability (Fig. 3) suitable for the desired therapeutical application.

Human breast adenocarcinoma cell line (MCF-7) was selected to evaluate the anticancer potential of calcidiol-NP and calcitriol-NP *in vitro*. Breast cancer is the most frequent cancer of women and its sensibility to vitamin D_3 active metabolites at nanomolar concentrations is well documented (29). Moreover, the presence and the activity of 1α-hydroxylase (CYP27B1) in MCF-7 cell line have been reported (30). The *in vitro* proliferation assay showed that the encapsulation of calcitriol and calcidiol does not modify their antiproliferative effects compared to free drugs. Four days after treatment initiation, similar responses were observed for free and encapsulated forms (Fig. 4). The advantage of NP was mainly noticed in the case of calcidiol with calcidiol-NP being significantly more efficient than free calcidiol ($P<0.05$) at days 7 and 10 as illustrated in Fig. 4. This higher activity was not surprising as calcidiol-NP present sustained release profile and very high stability in cell culture conditions (Fig. 2 and 3). In the case of calcitriol the advantage of NP was not very clear; calcitriol-NP presented higher efficacy than free form ($P<0.1$) after 10 days only. This lack of marked differences could be explained by the higher stability of free calcitriol in cell culture conditions compared to calcidiol and the rapid release profile of calcitriol-NP compared to calcidiol-NP. In the first case free calcidiol is less stable but the long lasting release from NP allowed the drug to be maintained at efficient concentrations. In the second case, the effectiveness of the treatment was mainly due to the higher stability of the free calcitriol rather than a modified release profile from NP and could explain the absence of significant differences between both free and encapsulated calcitriol treatments.

The benefit of NP could also be explained by the internalization of NP by tumor cells. Our *in vitro* investigations present the uptake of NR-NP by MCF-7 cells that indicate an improvement of the intracellular delivery of encapsulated drug compared to the free one. The uptake of NP by MCF-7 cells has been previously reported before by Jin *et al.*, using PLGA (poly($_{D,L}$) lactic-co-glycolic acid) based NP as carriers of paclitaxel (31).

Furthermore, the main interest of nano-encapsulation of vitamin D metabolites would be observed *in vivo*. Indeed, these NP would allow high accumulation in tumor and inside tumor cells that would not be achieved following injection of free forms. Intratumoral delivery could be a possible way of administration of calcidiol/calcitriol NP that facilitates tumor targeting and limits systemic side effects (32). The advantage of NP in intratumoral delivery was reported by Al-Ghananeem *et al.*, using rat mammary tumor as model. Paclitaxel-loaded NP induced a significant tumor growth inhibition compared to free form (33). Bernardi *et al.*, also reported that after intratumoral injection of indomethacin-loaded NP in implanted glioma model, NP are able to increase the intratumoral bioavailability and reduce tumor growth (34). We have previously reported that after intratumoral injection, polymeric NP remained in tumor and were engulfed by TAM (35). So, NP could exert long-lasting anticancer effects, maintain higher drug concentration and ensure large distribution inside tumors with limited systemic side effects. Future studies will be carried out *in vivo* to evaluate the calcitriol or calcidiol loaded NP anticancer activities and the impact of nano-encapsulation on calcemic effect. Once the concept of encapsulation of calcitriol has been validated, the targeting of cancer cells will be an interesting approach in order to increase the tumoral specificity.

CONCLUSION

Formulation parameters were studied and discussed using cholecalciferol to select the NP that present higher encapsulation efficiency, prolonged release and long stability. The advantageous role of oil in encapsulation was identified and explained. Calcidiol and calcitriol NP were successfully prepared with high encapsulation efficiencies and their release profiles as well

as their stabilities were characterized. These nanoparticles allowed maintaining the antiproliferative activities of encapsulated drug over 10 days against breast cancer cells. We can conclude that nano-encapsulation may offer a new and potentially effective administration strategy of vitamin D_3 that overcomes the actual limitations, and the targeted delivery of vitamin D_3 metabolites should be encouraged.

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