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Development of a nanoparticle-based system for the delivery of retinoic acid into macrophages

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ABSTRACT

The aim of the present work is to prepare nanoparticulate systems that can target and modulate the functions of mononuclear phagocytes by local administration. All-trans retinoic acid (RA) was chosen as an immunomodulator to be encapsulated in biodegradable nanoparticles (NP). Different formulations were prepared by the nanoprecipitation method and poly(D,L)lactic acid based nanocapsules (NC) were selected to continue the study. RA-NC demonstrated a sustained release profile and an enhanced stability for 7 days. The uptake of fluorescent (NileRed) labeled NP was conducted on bone marrow derived macrophages (BMM) *in vitro* and xenograft glioma nude mice *in vivo*. Fluorescent microscopy observations and flow cytometry analysis demonstrated that NR-NC were engulfed by BMM *in vitro* and lasted inside over 7 days. The intratumoral injection of NR-NC confirmed that NC were efficiently uptaken by infiltrated macrophages. The effects of RA loaded NC on BMM were also evaluated by RT^2-PCR array. Our results suggest that polymeric nanoparticles are suitable carriers to deliver RA into macrophages and can offer a new strategy in tumor macrophage-based treatment.

1. Introduction

Macrophages are innate immune cells that play a major role in the physio-pathology of numerous disorders that involve primary or secondary immune mechanisms. In particular, macrophages are crucial to the processes of tissue repair and form an important line of defense against infectious agents. On the other hand, macrophages are considered as playing detrimental functions under several conditions including chronic inflammatory diseases and tumors. Thus, in chronically inflamed tissue, infiltrating macrophages release cytokines/chemokines that lead to a sustained recruitment and activation of immune cells (Chellat et al., 2005). In malignant tumors, the so-called tumor associated macrophages (TAM) are thought to support both tumor progression and metastatic invasion (Coussens and Werb, 2002). Owing to their plasticity and involvement in a large range of pathologies, macrophages are thus considered as potential target in the design of innovative therapies (Ulbrich and Lamprecht, 2010; Watters et al., 2005).

Biodegradable polymeric nanoparticles (NP) are highlighted areas of drug delivery research. They are used to modify the release and distribution profile of active compounds allowing effective delivery to be improved and toxic effects to be lowered (Mora-Huertas et al., 2010). Moreover, polymeric nanoparticles increase intracellular drug delivery and its therapeutic effects *via* enhanced stability and sustained release especially for the drug that act *via* intracytoplasmic receptors. For example, Sahoo and Labhasetwar (2005) reported that NP enhanced paclitaxel efficacy on breast cancer cell line *via* sustained intracellular delivery. Bernardi et al. (2008) also presented the improvement of indomethacin cytotoxic effects on glioma cell line when it was nano-encapsulated. Besides, nanoparticle-based delivery system can be designed to target specific tissues, cells and/or intracellular compartments (Hillaireau and Couvreur, 2009). In this view, it has been long-lastly documented that polymeric nanoparticles without surface modification were preferentially taken up by macrophages following systemic or local administration. The adsorption of serum protein on polymeric-nanoparticle surface (opsonization) is an important factor which allows macrophages to recognize and internalize these particles. The opsonization and phagocytosis of NP are influenced largely by the NP physiochemical properties, in

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particularly the opsonization is increased with the augmented charge (Gessner et al., 2002) and hydrophobicity (Gessner et al., 2000) of NP surface. It was proved that decoration of NP surface with non-ionic and/or hydrophilic groups can decrease opsonization and hence limits phagocytosis (Owens and Peppas, 2006). As a consequence polymeric nanoparticles without surface modification are considered as ideal shuttles to deliver active drugs specifically into macrophages (Chellat et al., 2005; Ulbrich and Lamprecht, 2010). Such a strategy was applied to deliver antileishmanial agents into infected macrophages where nanoparticles allow specific delivery and maintenance of higher drug level inside macrophages (Basu and Lala, 2004).

All-trans retinoic acid (RA), one of the active derivatives of vitamin A, is a ligand of the retinoic acid intracytoplasmic receptors (RAR, RXR) and is known to influence the functions of macrophages. RA can inhibit the macrophage production of inflammatory cytokines and can enhance the secretion of suppressive cytokines as well (Pino-Lagos et al., 2008). It was proven also that RA altered the balance of TH1/TH2 type T cells through its impact on macrophages (Kang et al., 2007). Besides, RA has an influence on macrophages and dendritic cells in tumor. Such, RA inhibit the secretion of the angiogenic factors like VEGF and IL-8 by tumor-activated macrophages (Liss et al., 2002). Darmanin et al. (2007) suggested that RA can improve dendritic cell migration from the tumor to draining lymph nodes and may be boost the antitumor immunity. Poor water solubility and low stability of RA represent the main drawbacks in its dosage form formulations (Szuts and Harosi, 1991). The clinical use of RA was almost associated with rapid decrease of its serum concentration after continuous oral administration or intravenous injection (Achkar et al., 1994). It was documented that the encapsulation of RA can overcome these limitations and therefore offers more advantageous pharmaceutical forms. Ourique et al. (2008) showed that using nanocapsules enhanced RA photostability twofold than RA methanolic solution. Besides, Cirpanli et al. (2005) reported that RA-microspheres had a long chemical stability up to 4 months at 4 °C and lasted release profile over 11 days.

Intratumoral administration appears as an effective method for cancer chemotherapy and immunotherapy that allow to achieve ideal drug concentration in tumors and to limit systemic side effects (Goldberg et al., 2002). Previous reports have demonstrated that nanoparticle intratumoral delivery improves the anti-tumor efficacy. For example, Farokhzad et al. (2006) showed the advantage to use functionalized poly (D,L-lactic-co-glycolic acid)-block-poly(ethyleneglycol) NP in the intratumoral delivery of docetaxel to prostate cancer. Another team reported the enhancement of antitumor efficiency by using methoxy poly(ethylene glycol)-polycaprolactone core–shell NP for the intratumoral administration of cisplatin (Li et al., 2008) and docetaxel (Zheng et al., 2010). Moreover, the use of hyaluronan NP or poly (D,L-lactic-co-glycolic acid) NP for the intratumoral delivery of paclitaxel was also reported (Al-Ghananeem et al., 2009; Sahoo et al., 2004). Among these studies, non-modified surface or polyethyleneglycol (PEG) coated polymeric NP were used to target tumor cells. The PEG chains was utilized to avoid the uptake of intratumoral injected PEG-coated NP by non-tumoral cells. But in the case of polymeric NP without surface modification the uptake of polymeric NP by tumor associated macrophages (TAM) has not been investigated. It was well documented that non-modified surface NP were preferentially taken up by macrophages after opsonization *in vivo* (Owens and Peppas, 2006). So, it can be postulated that following intratumoral injection the polymeric NP could be taken up by TAM.

However, to the best of our knowledge, there is yet no study that attempted to design a NP-based system to deliver an immunologically active molecule into macrophages and, in particular, tumor-associated macrophages. Since in tumors, macrophages are continuously instructed by extracellular signals that shape their behavior, the goal of such a NP-based system would be to support a sustained deviation of macrophage functions that would counterbalance the effects of the macrophage microenvironment.

In this context, our goal was to design NP that meet the following criteria: (i) to be phagocytized by macrophages *in vitro*, (ii) to be phagocytized by tumor-associated macrophages *in vivo*, (iii) to efficiently encapsulate retinoic acid (RA), and (iv) to be biodegradable over a time-period of at least one week.

To achieve these aims, firstly nanospheres (NS) and nanocapsules (NC) formed with different biodegradable polymers (poly(D,L)lactic acid (PLA) and poly(ε-caprolactone) (PCL)) were compared regarding their ability to encapsulate RA. The selected nanoparticle formulation was then labeled by a fluorescent hydrophobic probe, NileRed (NR) to check the ability of such NP to be uptaken *in vitro* by primary cultures of bone marrow derived macrophages (BMM). Afterwards, an *in vivo* model of glioma was chosen to investigate the uptake of locally injected NP by TAM. Finally, the ability of nano-encapsulted RA to deviate the cytokine mRNA profile in bone marrow macrophages was verified.

2. Materials and methods

2.1. Materials

The biodegradable polymers used for NP formulation were poly(D,L)lactic acid (PLA, Mw 20,000 g/mol, Surmodics biomaterial, USA) and poly(ε-caprolactone) (PCL, Mw 14,000 g/mol, Sigma–Aldrich, St. Quentin Fallavier, France). NileRed and all-trans retinoic acid were purchased from Sigma–Aldrich. Montanox® VG 80 and Miglyol® 829 were purchased respectively from Seppic and Condea, France. C57Bl/6 mice (Charles River Laboratories, France) were used as BMM donors. For the tumor xenograft model, Athymic Nude-*Foxn1*^{nu} mice purchased from Harlan (Gannat, France) were used.

2.2. Preparation of NP

Retinoic acid loaded nanoparticles were prepared by using the nanoprecipitation technique (Fessi et al., 1989). Briefly polymer and RA were dissolved in acetone at a concentration of 0.50% and 0.010% (w/w) respectively. To form the core of nanocapsules, organic oil (miglyol® 829 at 0.75%) was added to the solution previously described. This organic phase was then poured into an aqueous phase containing 0.05% of Montanox® VG 80 as surfactant. Nanoparticles (NS or NC) were instantaneously formed by the rapid solvent diffusion inducing polymer precipitation. The nanoparticle suspension was stirred moderately at room temperature for 20 min. Acetone and a part of the water were removed under vacuum using a rotary evaporator (Rotavapor® RE-140, Büchi, Switzerland). Fluorescence labeled NP were prepared in the same way by incorporating NileRed in the organic solution at a concentration of 0.005%. Similar formulas without any active compounds were prepared as controls (blank-NP). The nanoparticle suspensions were stored at 4 °C in hermetically closed vials. Before the *in vivo* application, the NP suspension was centrifuged at 18,000 × *g* for 20 min and the particles were redispersed in a solution of phosphate buffered saline (PBS, 0.1 M, pH 7.4) containing 0.1% of Montanox® VG 80 to obtain a stable suspension with physiological values of pH and osmolarity. The obtained suspension was then sterilized by filtration through 0.2 μm syringe filter (Minisart®, Sartorius, France). All formulations were made in triplicate.

2.3. Characterization of NP

The particle size, size distribution and zeta-potential were measured using photon correlation spectroscopy (Malvern® Zetasizer nano-series) using nanoparticle suspension diluted in deionized water at room temperature. The results were recorded as the average of three measurements. The size distribution is given by polydispersity index (PdI). Formulations with a PdI smaller than 0.2 were accepted. Surface morphology of blank-NP before and after the dispersion in PBS was observed using a scanning electron microscope (SEM, Hitachi S800) at an accelerating voltage of 15 kV after coating the nanoparticle surface with gold–palladium layer under vacuum with a cathodic pulverizer technics Hummer II (6 V, 10 mA).

2.4. Encapsulation efficiency

The total drug (RA or NR) 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. Non-encapsulated active (free) was determined in the supernatant after ultracentrifugation at 18,000 × *g* for 20 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.

RA was analyzed by RP-HPLC validated method using Waters 600 Controller and Waters 717 Auto sampler (Waters, France) on a Kinetex C18 column (2.6 μm, 4.6 mm × 100 mm). An isocratic mobile phase consisting of 35% of acetonitrile, 30% of methanol and 35% of 0.5% acetic acid solution was used with a flow rate of 1.4 ml/min. The photodiode-array detector (Waters 2996) was adjusted at λ = 356 nm. A 10 μl aliquot sample was injected. The calibration curve for the quantification of RA was linear over the range of concentrations used, from 0.05 μg/ml to 10 μg/ml (r^2 = 0.999). The determination of NR was also performed by the Waters RP-HPLC using a X-Terra MS C18 column (5 μm, 4.6 mm × 150 mm) and an isocratic mobile phase consisting of methanol/water (80:20). This method was modified from Lamprecht and Benoit (2003).

2.5. In vitro RA-NP release studies

A dialysis method was used under Sink conditions to evaluate the release profile of RA loaded NP. 1 ml of RA-loaded NP suspension was filled into a dialysis tube (MWCO 6000-8000, Cellu-Sep®) and incubated in 100 ml of ethanol: phosphate buffer solution (PBS, pH 7.4) (2:8) under moderate magnetic stirring (150 rpm) at 37 °C. At predetermined intervals of times, samples of 0.5 ml were withdrawn, replaced by 0.5 ml of fresh medium and the drug released from NP was quantified by RP-HPLC. Three experiments were conducted and free-RA solution was used as control to estimate the RA diffusion rate through the membrane.

2.6. RA-NP stability test

To study the stability of encapsulated RA in cell culture conditions, RA loaded NP were suspended in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, France) supplemented with penicillin–streptomycin (1 μg/ml, Invitrogen, France) in order to obtain a RA concentration of 10 μg/ml. Free RA was used as control at the same concentration. All samples were incubated at 37 °C for 7 days. Each day, 0.5 ml was taken and was analyzed by RP-HPLC to determine the remained quantity of RA. The study was achieved in triplicate.

2.7. Generation of murine bone marrow macrophage (BMM)

Bone-marrow-derived macrophages (BMM) were cultured as previously described (Davoust et al., 2006). Briefly, bone marrow cells were flushed from the femur and tibia of 2–5 month-old female C57Bl/6 mice and cultured for 7 days at a density of 5×10^5 cells/ml in IMDM supplemented with 15% Fetal Clone II (Perbioscience, France), penicillin–streptomycin (1 μg/ml), and 10 ng/ml macrophage-colony stimulating factor (M-CSF, Tebu-bio, France).

2.8. In vitro cellular uptake of NP

BMM were incubated with NR-labeled NP, blank-NP and free NR at a final concentration corresponding to 0.4 μg/ml of NR in the culture medium. After 24 h of incubation the media was replaced by serum-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, France) supplemented with B-27 and N-2 (Invitrogen, France). The phagocytosis of NP was assessed by fluorescence microscopy (Ziess, Axio-Imager. Z1) at 24 h, 72 h and 7 days and by confocal laser microscopy at 24 h (Leica TCS SP2). BMM were fixed in 4% paraformaldehyde (Invitrogen, France) for 10 min at room temperature and washed three times in PBS. Cells were incubated for 30 min at room temperature with a blocking solution consisting of 4% bovine serum albumin (BSA, Invitrogen, France) diluted in PBS and supplemented with 10% normal goat serum (Invitrogen, France). Cells were then incubated overnight at 4 °C with a rat anti-mouse F4/80 monoclonal antibody (Abcys, France) at 1:50 dilution in blocking solution. Cells were then rinsed three times in PBS and incubated with a biotinylated goat anti-rat antibody (Invitrogen, France) for 1 h at room temperature. After three washes, cells were finally incubated with streptavidin Alexa Fluor 488 (Invitrogen, France) diluted 1:100 in PBS. 4′,6′-diamidino-2-phenylidole (DAPI, Invitrogen, France) staining of nuclei was performed and slides were mounted using Fluoroprep (Bio-Mérieux, France). A quantitative analysis of phagocytosis was made by flow cytometry (488 nm argon ion laser, BD FACSCantoII™) at 24 h, 72 h and 7 days. BMM were detached by trypsin treatment and fixed in 1% paraformaldehyde. The fluorescence intensity was measured in FL2 and the intensity of side scattering channel (SSC) was also observed.

2.9. Intratumoral injections of NP in a xenograft glioma model

To investigate *in vivo* the phagocytosis of NP by tumor-infiltrating macrophages, a human xenograft glioma model developed by Taillandier et al. (2003) was used in accordance with French animal care guidelines and with the approval of the animal ethical committee CREEA of Lyon. Briefly, a glioblastoma multiforme (10 mm^3 fragment) was subcutaneously implanted in the right posterior inguinal pit of nude mice. When tumors had an average volume of 1 cm^3 (about 3 weeks after inoculation), mice were divided in two groups of three to be given either NR-labeled NP or blank-NP dispersed in PBS by intratumoraly injection of 200 μl containing 2.5 mg of nanoparticles (100 mg of NP/kg). Mice were euthanized 48 h post NP injection and tumors were collected for histological analysis. Tumor tissues were fixed with 4% *paraformaldehyde*, *cryoprotected in sucrose*, *frozen* and sectioned into 10 μm slides. Slides were incubated with a blocking solution consisting of 4% BSA diluted in PBS and supplemented with 10% normal goat serum. Then, they were incubated overnight at 4 °C with a rabbit anti-mouse Iba-1 monoclonal antibody (Wako, Japan) at 1:1000 dilutions in the blocking solution. Biotinylated goat anti-rabbit antibody (Invitrogen, France) was applied as the second antibody for 1 h at room temperature. After three washes, slides were incubated with streptavidin Alexa Fluor 488 diluted 1:100 in PBS. DAPI staining of nuclei was performed and slides

Table 1
Physicochemical characteristics of RA-loaded nanoparticles.

Sample	Polymer	Form	Particle mean size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency%
E1	PCL14000	NS	177.7 ± 1.7	0.08 ± 0.02	−18.5 ± 0.4	55.6 ± 9.3
E2	PCL14000	NC	229.8 ± 5.6	0.16 ± 0.01	−29.4 ± 0.6	84.9 ± 1.3
E3	PLA20000	NS	153.6 ± 2.9	0.14 ± 0.02	−10.4 ± 1.0	10.4 ± 1.3
E4	PLA20000	NC	192.7 ± 7.3	0.09 ± 0.01	−21.6 ± 5.4	90.2 ± 0.8

were mounted using Fluoroprep. The observation was assessed by fluorescence microscopy (Ziess, Axio-Imager. Z1).

2.10. Evaluation of RA-loaded NP effects on BMM (RT^2-PCR array)

BMM were cultured at 5×10^5/ml and incubated with RA-NP (dilution 1/100) for 24 h followed by media changing. Blank-NP were used as control. We evaluated the modifications of BMM cytokine gene expression 24 h after media changing by Mouse Common Cytokines RT^2 Profiler™ PCR Array (PAMM-021, SABiosciences Corporation®) which profiles the expression of 84 important cytokines. Total RNA was isolated from two independent cultures of BMM and reverse transcribed according to the manufacture specifications. Complementary DNA was amplified by polymerase chain reaction (PCR) for 40 cycles after an initial denaturation cycle of 10 min at 95 °C. Each amplification cycle consisted of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. The results were normalized with 5 housekeeping genes (Gusb, Hprt1, Hsp90ab1, Gapdh and Actb). Fold change between RA-loaded NP treated BMM and blank-NP treated BMM was determined using the manufacturer software according to the comparative Ct method. Data from genes with Ct value >30 or fold change <2 were considered as non-significant.

3. Results

3.1. Development of RA loaded nanoparticles

Four nanoparticle formulations were prepared in triplicate and characterized by their mean size, their size distribution, their zeta potential and their encapsulation efficiency as presented in Table 1. All formulations had a negative charge due to the presence of polymeric terminal carboxylic groups on the NP surface. Nanospheres E1 and E3 showed a small particle size (177.7 ± 1.7 and 153.6 ± 2.9 nm respectively) compared to the nanocapsules E2 and E4 (229.8 ± 5.6 and 192.7 ± 7.3 nm, respectively) due to the presence of oil in NC contrarily to NS. Moreover, nanocapsules presented higher encapsulation efficiency than nanospheres. Therefore, nanocapsules (E2 and E4) were selected to continue our study.

The *in vitro* release and the stability of RA were evaluated and the behavior of PCL-NC (E2), PLA-NC (E4) and RA ethanolic solution was compared. The results of *in vitro* release are shown in Fig. 1A. RA in ethanolic solution passed through the membrane rapidly and totally during the first 9 h which refer to the RA diffusion rate through the membrane. Encapsulated RA release pattern showed a rapid release of 50 ± 3% and 55 ± 2% of initial RA content in E2 and E4 respectively during the first 24 h and a sustained release profile up to 80% over 4 days (Fig. 1A). No significant difference was observed between the release profile of PCL-NC (E2) and PLA-NC (E4).

The stability of free and encapsulated RA in cell culture conditions is shown in Fig. 1B. A rapid degradation (40%) of free RA was observed after 24 h and only 16 ± 6% of free RA remained in the IMDM after 96 h. In contrast, encapsulated RA degraded slower with only 10% degradation after 24 h. At the end of the study (7 days) PCL-NC (E2) showed higher RA stability than PLA-NC (E4). About 48 ± 4% of RA remained in E4 after 7 days compared to 66 ± 2% for E2.

However, based on the encapsulation efficiency and the size distribution, we selected PLA-NC (E4) to continue our study.

3.2. NP preparation for the uptake study

Based on the selected formulation (E4), unloaded nanocapsules (blank-NC) and NileRed labeled nanocapsules (NR-NC) were prepared and characterized. NC properties are presented in Table 2. The mean size of blank-NC was 187.7 ± 6.2 nm with a narrow size distribution and a polydispersity index of 0.08 ± 0.03. The surface of NC was negatively charged with a zeta potential value of −15.4 ± 2.1 mV. When blank-NC were redispersed in PBS (blank-NC-PBS) no significant changes on particle size or polydispersity were observed. The NC zeta potential decreased to −25.3 ± 1.4 mV while the pH changed from 3.29 to 6.95 due to the buffer properties of PBS (pH 7.4). The pH increase caused the deprotonation of carboxylate groups and the decrease of zeta potential as already mentioned by Trimaille et al. (2003). Moreover, the SEM observations of blank-NC, illustrated in Fig. 2, revealed that all nanocapsules were spherical with no large pores. In accordance with data obtained by the dynamic light scattering instrument the SEM images of blank-NC showed that NC had monodispersed size near to 200 nm. The redispersion in PBS had no substantial effects on the morphological aspect of NC but the picture quality was decreased probably due to the precipitation of PBS salts during the sample preparation.

NR-labeled NC had the same size distribution with a mean size of 198.1 ± 11.4 nm. The same zeta potential change of NR-NC was noticed after redispersion in PBS. The encapsulation efficiency of NR was 91.2 ± 3.7% and 80.3 ± 1.5% before and after redispersion in PBS, respectively.

3.3. In vitro NR-NC uptake

Bone-marrow-derived macrophages were incubated with NR-labeled NC, blank-NC and free NR for 24 h followed by media changing. The uptake study of NP was assessed by fluorescence microscopy at 24 h, 72 h and 7 days and by confocal laser microscopy at 24 h while no toxic effects were observed on BMM. It can be observed that no fluorescence was detected in BMM treated by blank-NC (Fig. 3A). For NR-NC treated BMM confocal microscopy pictures confirmed the intracellular localization of NR-NC as shown in Fig. 3B. Fluorescence microscopy images at 24 h, 72 h or day 7 post-treatment showed that the intracytoplasmic granular fluorescence remained in BMM for 7 days (Fig. 3C).

To confirm these data, a flow cytometry analysis was conducted on BMM incubated with free-NR, blank-NC or NR-NC. Results of the fluorescence intensity measurement in FL2 (Fig. 4A and B) were in accordance with microscopy observations. Free-NR treated BMM showed low fluorescent intensity after 24 h with mean fluorescence intensity (MFI) of 207 compared to 172 for the non-treated ones (control), probably due to the rapid elimination of NR from macrophages. In contrast, after 24 h, MFI of NR-NC treated BMM was 4 fold higher than control ones and this higher fluorescence intensity lasted over 7 days with MFI = 752 and 406 at 24 h

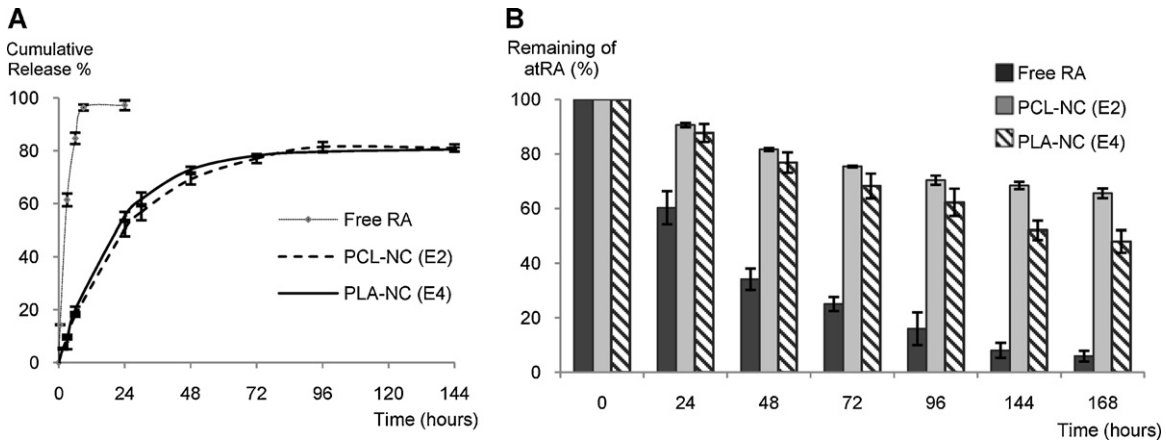

Fig. 1. (A) *In vitro* E2 (PCL-NC) and E4 (PLA-NC) release profile assessed by dialysis method. Free-RA solution was used as control. (B) Results of stability study of encapsulated RA in the cell culture conditions for 7 days. Free RA was used as control. E2 or E4 were dispersed into 10 ml of IMDM and incubated at 37 °C.

Table 2
Physicochemical characteristics of nanoparticles for *in vitro* and *in vivo* uptake studies.

Sample	Particle mean size (nm)	Polydispersity index	Zeta potential (mV)	pH	Entrapment efficiency (%)
Blank-NC	187.7 ± 6.2	0.08 ± 0.03	−15.4 ± 2.1	3.29 ± 0.10	–
Blank-NC-PBS	181.9 ± 0.4	0.06 ± 0.04	−25.3 ± 1.4	6.95 ± 0.01	–
NR-NC	198.1 ± 11.4	0.11 ± 0.02	−20.9 ± 2.8	3.24 ± 0.11	91.2 ± 3.7
NR-NC-PBS	188.8 ± 1.5	0.05 ± 0.02	−27.4 ± 2.3	6.93 ± 0.02	80.3 ± 1.5

and 7 days respectively. Blank-NC treated BMM showed the same fluorescence intensity level than control. So there were no significant changes in the case of free-NR or blank-NC and only NR-NC increased the macrophage MFI significantly.

Moreover, in NR-NC or blank-NC treated BMM, changes in BMM granularity were observed as assessed by the mean values in side scattering channel (SSC) intensity (Fig. 4C and D). Such an increased granularity was maintained over a period of 7 days indicating that, following phagocytosis, NC were not fully degraded in the next 7 days and remained in the endosomal compartment of macrophages.

3.4. *In vivo* NR-NC uptake

To assess PLA based NC uptake by macrophages *in vivo* NR-NC or blank-NC dispersed in PBS were injected intratumoraly in nude mice bearing xenograft glioma. Observations under fluorescence microscopy (Fig. 5) showed that in tumors injected with NR-NCs, fluorescent granular material could be detected and was localized exclusively within Iba1-labeled macrophages. No fluorescence could be observed in tumors injected with blank-NC (data not shown). These results indicated that NR-NC were efficiently internalized by macrophages but not by glioma cells or tumor-associated vessels. Interestingly, NR-NC containing macrophages were localized far away from the injection site, notably in perivascular locations, in the outer margins of the tumor (Fig. 5A and C) or, occasionally within the tumor parenchyma itself (Fig. 5B).

3.5. *RA-NC modulate cytokine gene expression of cultured BMM*

To investigate the effects of RA-NC on macrophage functions *in vitro*, a quantitative RT^2 Profiler^{TM} PCR cytokine array was used, allowing alterations of cytokine gene expression to be assessed in RA-NC treated BMM as compared to blank-NC treated BMM. Results were filtered so that only genes showing a robust expression (detectable below 30 cycles) and an expression fold change of at least 2 were considered as significant. Under these experimental

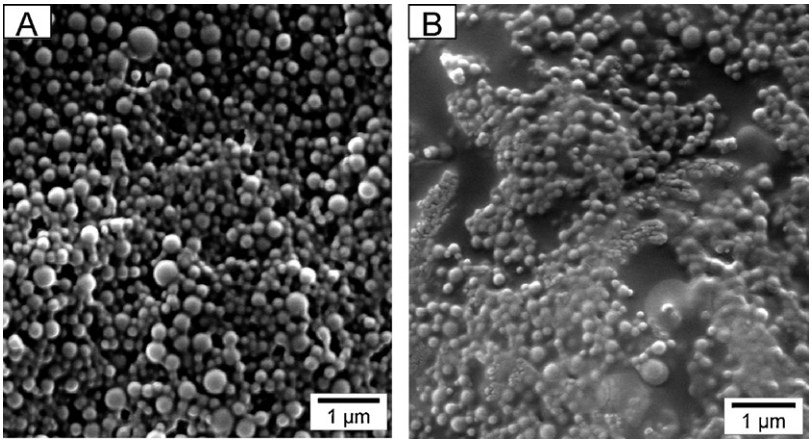

Fig. 2. Scanning electron microscopy (SEM) images. SEM showed the morphology of blank-NC (A) and blank-NC-PBS (B).

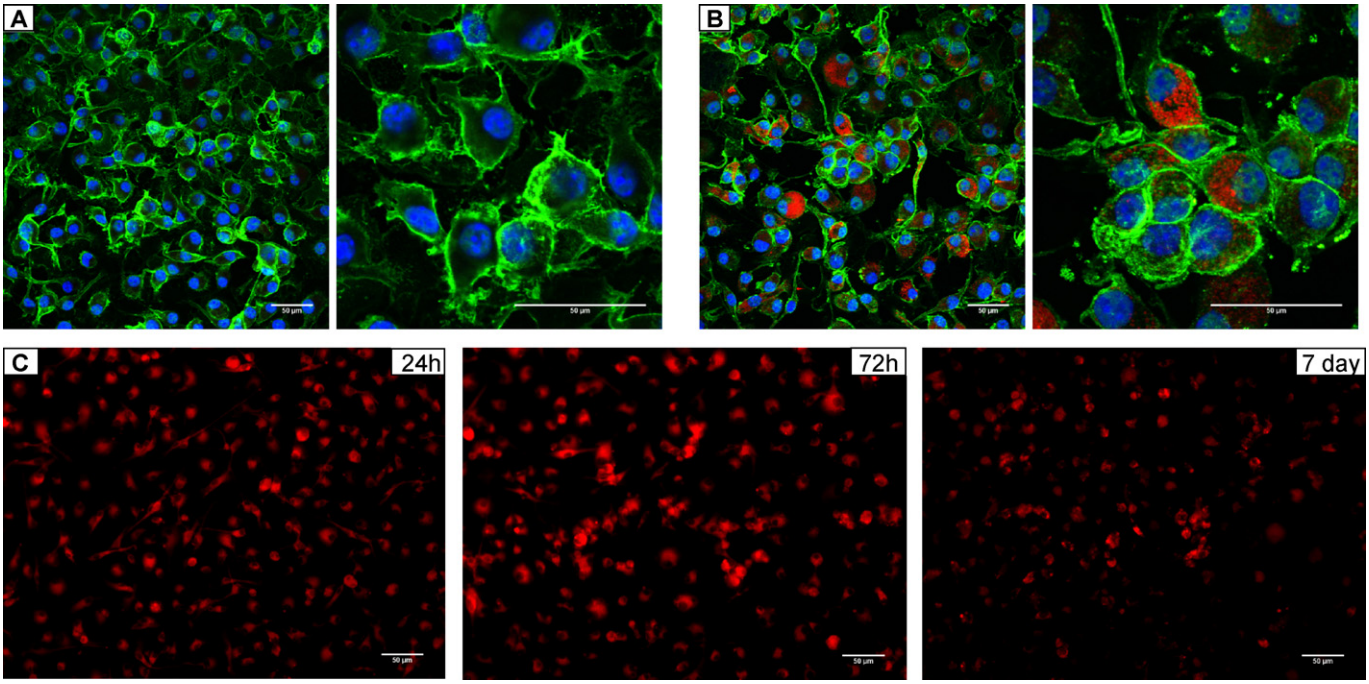

Fig. 3. *In vitro* uptake study of NR-NC by BMM. (A and B) Confocal microscopy images of blank-NC (A) or NR-NC (B) treated BMM (bar = 50 μm). (C) Fluorescent microscopy images in red channel of NR-NC treated BMM at 24 h, 72 h and 7 days (bar = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

conditions, we found that Bone morphogenetic protein 2 (BMP-2) gene expression was increased by a mean factor of 3.37 in RA-NC treated BMM as compared to blank-NC treated BMM (Fig. 6). This result indicates that RA-NC phagocytized by BMM were able to bias the cytokine profile of macrophages.

4. Discussion

Biodegradable polymeric nanoparticles are a promising strategy in the drug delivery development since they can offer more stability, modified release and specific delivery of drug. In our study a nanoprecipitation technique was used to encapsulate RA in nanoparticles with two different internal structures: nanospheres (NS), which are matrix systems and nanocapsules (NC), vesicular systems in which the oily cavity is surrounded by a thin polymeric membrane. Differences in size, encapsulation efficiency and release profile were expected from these two forms. Moreover two polymers were also used in this study; poly(ε-caprolactone) (PCL, Mw 14,000) and poly(D,L)lactic acid (PLA, Mw 20,000). Both are synthetic polyester polymers approved by the Food and Drug Administration (FDA) with unique properties of biocompatibility and biodegradability. Indeed, they are hydrolyzed to nontoxic metabolites in physiological conditions. However, they differ by their degradation rates, their hydrophobicity and their crystalline states (Holland et al., 1986).

The encapsulation of RA was successfully achieved and nanocapsules showed higher encapsulation efficiency than nanospheres. This could be explained by the role of NC lipophilic core (Miglyol® 829) that increased the quantity of RA dissolved and then improved its entrapment (Couvreur et al., 2002). PCL-NC (E2) and PLA-NC (E4) presented similar release profiles of encapsulated RA. These release profiles were composed of a rapid phase due to the non-encapsulated RA and the release of surface-adsorbed RA (20% during 6 h) followed by a sustained release phase over 4 days (Fig. 1A). The incomplete release (80% of RA released after 7 days) could be attributed to RA deeply entrapped in the polymeric network which needs more polymer erosion to diffuse outside nanoparticles. The incomplete release of encapsulated RA was also reported by Jeong et al. (2004) for RA core–shell nanoparticles of poly(ε-caprolactone)/poly(ethylene glycol) diblock copolymer where 80% of RA was released after 16 days. An outstanding result is that the encapsulation of RA considerably improved its stability in the cell culture conditions (Fig. 1B) and this give the possibility that NC could maintain RA effects on the macrophages for a longer period. Moreover, it was shown that PCL-NC were more stable than PLA-NC probably because of the difference in degradation profile and hydrophobicity between the two polymers, PCL being more hydrophobic and having a lower degradation rate. For example, Leroueil-Le Verger et al. (1998) have previously investigated the degradation of biodegradable polymer-based nanoparticles during *in vitro* release studies and have observed a higher degradation rate for PLA than for PCL (molecular weigth loss of 10% and 3% for PLA and PCL, respectively).

From all the results above, PCL and PLA based NC were considered as good carriers for RA. The sustained release profile and the relatively good stability of encapsulated RA appeared as suitable for a potential therapeutic application on macrophages. The E4 (PLA-NC) was selected to continue the study because it displayed the highest encapsulation efficiency (90.2%) and a narrow size distribution, with a mean size smaller than 200 nm which is adapted to a sterilization process by filtration.

Our goal was to develop a NP-based system to deliver an immunologically active molecule into macrophages and, in particular, tumor-associated macrophages. In this scheme, to achieve therapeutic effects, NP have first to be phagocytized by macrophages and secondly must release the biologically active molecule that can trigger macrophage activation. In this view, *in vitro* investigations of NR-labeled NC provided important data about the uptake of NC by macrophages. BMM were incubated with NR-NC or blank-NC for 24 h and labeled as described in the materials and methods part. 24 h of incubation was chosen to obtain a maximal internalization as documented by other groups (Kanchan and Panda, 2007). We postulated that the hydrophobic properties of PLA-NC surface could enhance the internalization of particles by

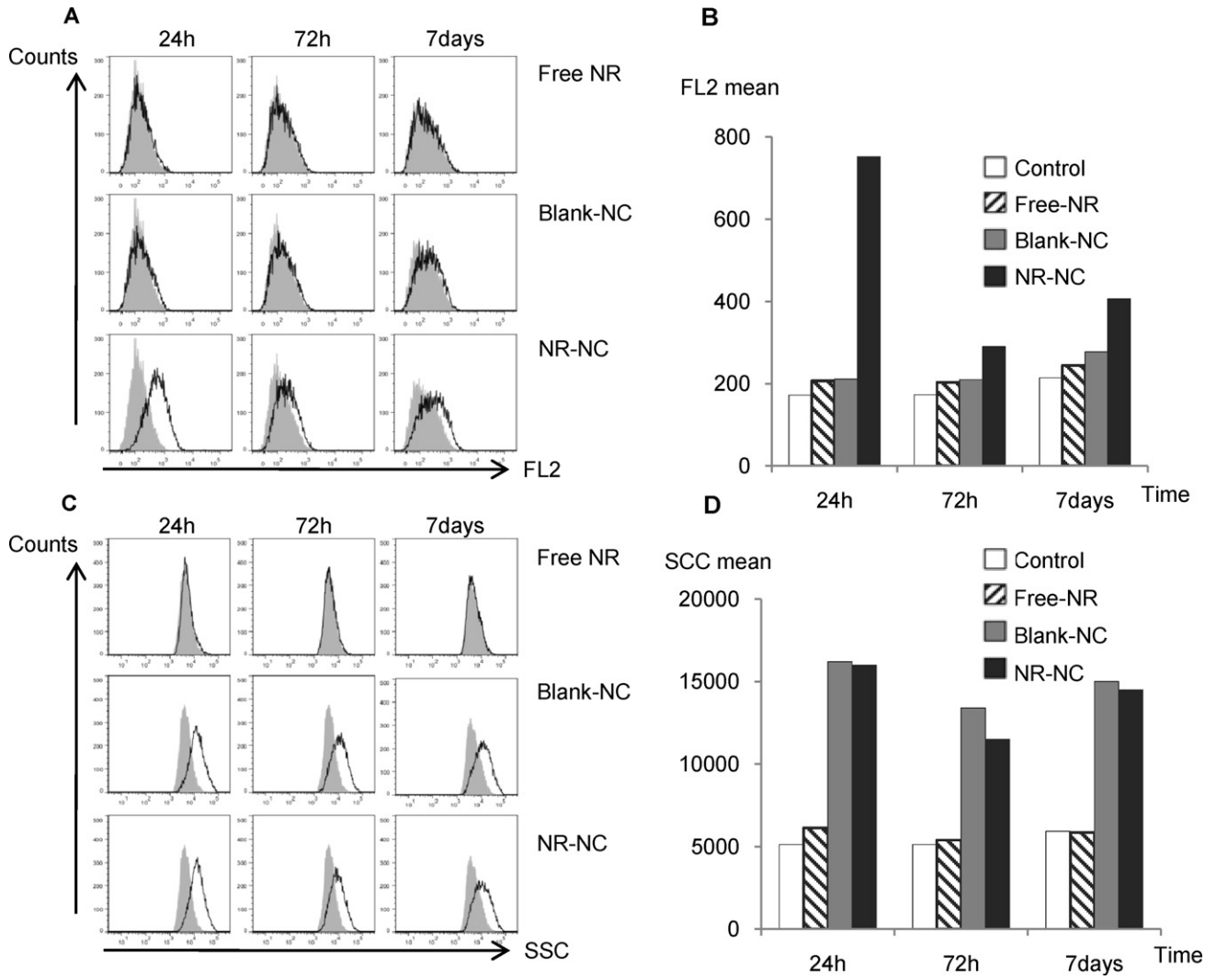

Fig. 4. *In vitro* uptake of NR labeled NC performed by flow cytometry. Non-treated BMM was used as control. Histograms show the mean of fluorescence intensity in FL2 channel (A and B), and the mean of side scattering channel intensity (C and D).

macrophages *via* serum adsorption (Chellat et al., 2005). Thanks to NC-properties the NR release was sustained and its degradation was limited inside the macrophages. Indeed, the fluorescence microscopy pictures (Fig. 3C) proved the internalization of NR-NC by macrophages and their stability inside them over 7 days. Moreover, the flow cytometry measurements confirmed microscopy observations and allowed to quantify both the fluorescence intensity and the macrophage granularity. The increase of SSC intensity mean for NC-treated macrophages gave another proof of phagocytosis or, at least, internalization of NC by macrophages. Similar shifts of cell granularity were previously described by Xia et al. (2008) who studied the internalization of magnetic nanoparticles by hepatoma cells, and by Coester et al. (2006) about the uptake of gelatin-NP by dendritic cells. Kanchan and Panda (2007) also recognized the enlargement of cell size after incubation of murine macrophage cell line J774 with PLA nanoparticles. Therefore, these studies along with our results indicate that PLA-based NC are suited carriers for drug delivery to macrophages and they are able to sustain drug effects on macrophages.

We selected glioma (the most frequent brain tumor) as a tumor model to study NP uptake by TAM *in vivo*. The infiltration of glioma by macrophages is well documented (Badie and Schartner, 2000) and it was proven that TAM conserve their phagocytosis capacity (Nickles et al., 2008). Thus; TAM represent a potentially valuable target in glioma for therapeutic applications (Alizadeh et al., 2010). In this context, we found that NR-NC injected *in situ* were efficiently phagocytized by glioma-infiltrating macrophages but not by glioma cells (Fig. 5). These results are reminiscent of those described with cyclodextrin-based nanoparticles injected in glioma-bearing mice (Alizadeh et al., 2010). Interestingly, in that study and ours, NP accumulated in the periphery of glioma tumor but not in tumor peri-necrotic area as reported in the case of intravenous injection of poly (L-glutamic acid)-macromolecules (Melancon et al., 2010). Our findings suggest that the intratumoral injection of non-modified surface polymeric NP is a promising way to target TAM and may allow the development of anti-tumor treatment based on modulation of TAM functions.

To further assess this strategy, the ability of RA-NC to modulate macrophage functions *in vitro* was investigated by a RT^2 Profiler™ PCR cytokine array (Fig. 6). The results showed that bone morphogenetic protein 2 (BMP-2) gene was over-expressed in BMM treated with RA-NC. BMP-2 is a member of the transforming growth factor b (TGF-b) superfamily (Hogan, 1996) and plays an important role in the pro-apoptotic activity of retinoids in tumors (Merino and Hurle, 2003). Hallahan et al. (2003) reported the mediated role of BMP2 in the medulloblastoma cell apoptosis induced by retinoids and demonstrated that secreted BMP-2 might induce apoptosis in retinoid-resistant medulloblastoma cells. It can be postulated that

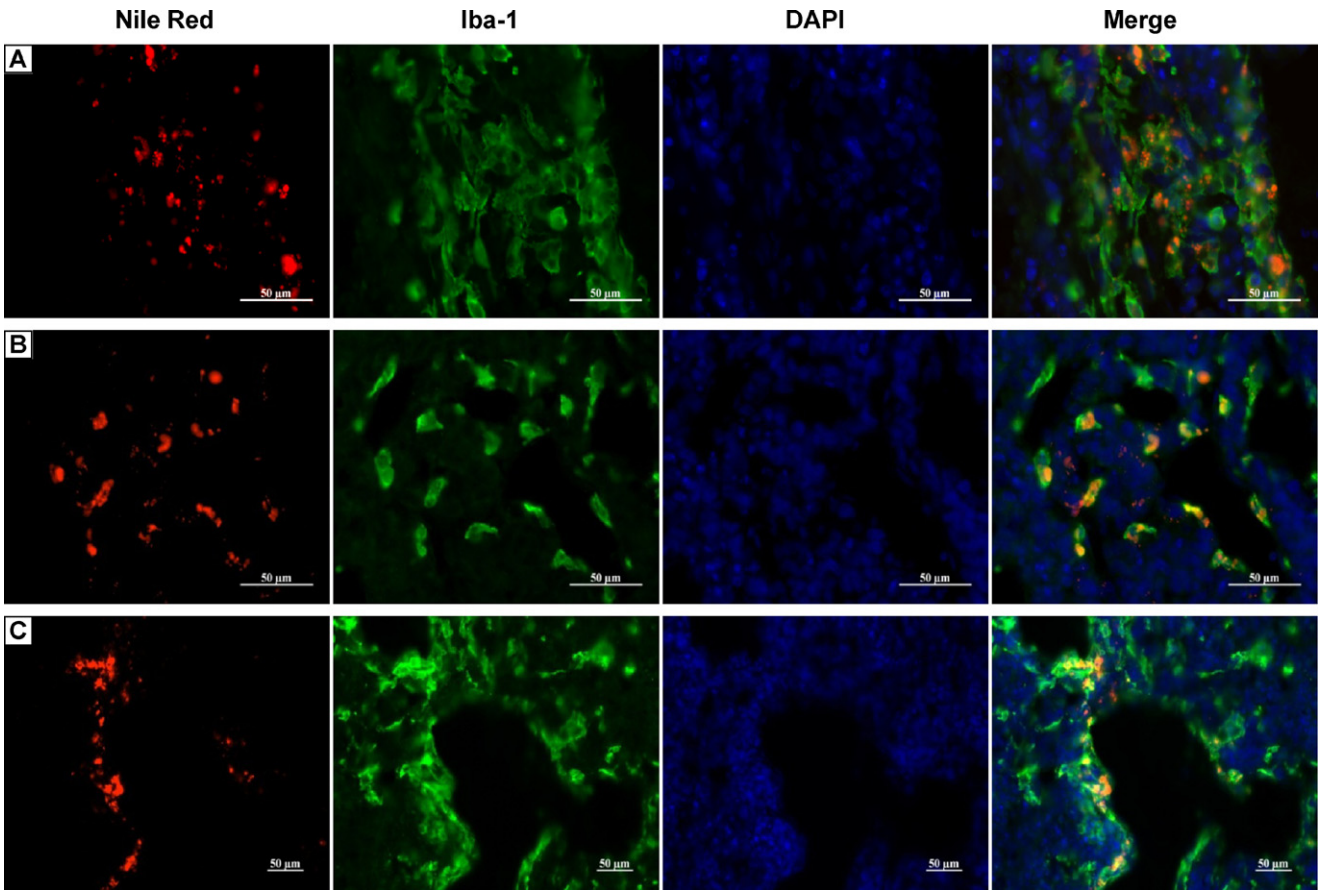

Fig. 5. *In vivo* NR-NC uptake by tumor infiltrated macrophages after 48 h of NC intratumoraly injection. Nanoparticles were labeled by NileRed (red), macrophages were immunolabeled by Anti-Iba1 monoclonal antibody (green) and DAPI (blue) was used for staining the nucleus. NR-NC were localized within the macrophages at the edge of tumor (A), inside the tumor (B) and around the vessel (C) (bar = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

after RA-NC intratumoral injection, these NC will be phagocyted by TAM and the intracellular release of RA may enhance the BMP-2 secretion and allow apoptosis induction of tumor cells. Future studies have to be developed to verify the possibility of BMP-2 secretion from RA-NC treated macrophages in tumor microenvironment.

On the other hand, it is known that retinoic acid acts as an anti-tumoral agent in different tumors like gliomas (Defer et al., 1997), medulloblastoma (Hallahan et al., 2003), and others (Sun and Lotan, 2002). Moreover, Dou et al. (2007) demonstrated that after NP internalization, the nano-encapsulated drug can be released from macrophages into extracellular space. Soma et al. (1999) demonstrated also the anti-tumor efficacy of nano-encapsulated doxorubincine after the uptake by macrophages. For these reasons, we may also suggest that besides immunomodularity effects exerted on macrophages, RA released from TAM into the tumor environment may exert long-lasting anticancer effects that would not be achieved following injection of either free RA or RA-loaded NC that do not target macrophages.

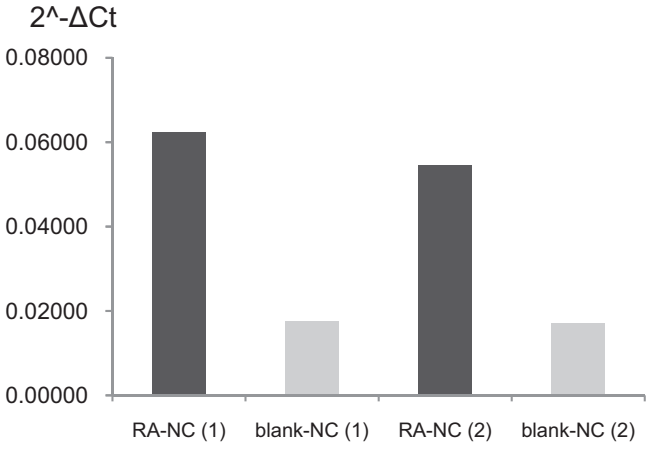

Fig. 6. *In vitro* gene expression changes of RA-NC treated macrophages evaluated by RT^2 Profiler™ PCR Array in duplicate. The Bmp-2 gene expression was three times higher in RA-NC treated BMM than blank-NC treated BMM calculated by Ct method.

5. Conclusion

Retinoic acid was successfully encapsulated in polymeric nanoparticles allowing an increase of its stability and the specific targeting of macrophages. PLA based NC were highly taken up and maintained inside the macrophages *in vitro* at least 7 days. *In vivo* study confirmed that intratumoral-injected NC were preferentially engulfed and maintained inside TAM 48 h after injection. Moreover, NC had a sustained RA-release profile over 4 days and enhanced RA-stability. Thus, PLA based NC are a suitable form to deliver RA into macrophages and can offer a new strategy in tumor treatment where macrophages are known to play a major role.

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