In Vitro Alendronate Delivery Using Liposomal Iron Oxide Nanoparticles

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ABSTRACT

Because to their magnetic properties, superparamagnetic iron oxide nanoparticles (SPIONs) might be considered as a good delivery vehicle after grafting of their surface with a therapeutical drug. On the other hand, drug encapsulation into liposomes allows protection of the agent against enzymatic degradation, reduces the dose and toxics effects; however, a selective and controlled release of the drug out of the vesicles is still difficult to achieve. A combination of both properties (magnetic targeting and drug encapsulation) is proposed to deliver an anticancer drug, alendronate (an hydroxymethylene bisphosphonates molecule). Two human cancer cell lines, A431 and U87-MG, have been tested in vitro with various alendronate formulations: on both cell lines, an effective 100% cytotoxic effect could only be achieved with alendronate grafted-SPION entrapped into liposomes and within a magnetic field.

Keywords: alendronate, superparamagnetic iron oxide nanoparticles, liposome, drug delivery

1 INTRODUCTION

Bisphosphonates (particularly 1-hydroxymethylene-1,1-bisphosphonate) have been used for decades in the standard therapy of bone-related diseases, including bone metastasis of various malignancies [1], and they might as well be toxic on early cancer cells themselves [2]. However, while these molecules have been used extensively, giving very valuable clinical results, they have also shown a non-negligible number of side effects that could prevent their use [3, 4]. Then, one can consider developing new galenic formulations for these compounds in order to allow a better delivery and less toxicity. Various strategies are used as development of bisphophonates prodrugs with a lower bone mineral affinity [5], encapsulation in liposomes [6,7], vectorization through nanocrystals functionalization [8, 9]

Indeed, liposomes are bubbly self-closed lipid vesicles formed by one or several concentric lipid bilayers with an aqueous phase inside or between bilayers. They are biocompatible and can entrap pharmaceutical agents, protecting them from external effects and allowing to locally deliver them in a concentrated form [10]. However, *in vivo* studies have always needed major improvements on this simple system, in order for these liposomes to successfully deliver their payload to specific targets (for example by grafting on their surface polymers that will allow them to escape the reticulo-endothelial scavenging pathway) [11].

Superparamagnetic iron oxide nanoparticles have emerged as one of the primary nanomaterials for biomedical applications such as cancer therapy and diagnosis [12,13]. This is related to their low toxicity and according to their nanometer size scale to their specific magnetic properties at room temperature. Although the use of SPIONs as magnetic resonance imaging contrast agents is established, their potential as drug targeting and drug delivery agents is still in the early stage of evaluation. The nanoparticles can be coated by drugs and can be targeted to a desired treatment location by externally localized magnetic steering.

A combination of both properties of liposomes and SPIONs is proposed to deliver *in vitro* a bisphosphonate drug: alendronate. Two human cancer cell lines, A431 and U87-MG were treated with various drug formulations, from alendronate alone to alendronate grafted on an SPION entrapped in liposome and placed in a magnet field. The SPION is a γFe_2O_3 nanocrystal. Cytotoxicity measurements showed that the best condition (100% of cell growth inhibition) was obtained on both cell lines using the lattest formulation with a magnet. Flow cytometry measurement of the drug cell uptake was correlated with this result.

2 MATERIALS AND METHOD

2.1 γFe_2O_3@Alendronate nanocrystals, Rhodamine B Coupling And Characterization

The synthesis of γFe_2O_3 nanocrystals and their surface coating with 1-hydroxymethylene-1,1-bisphosphonate molecules was described previously[8,9]. Briefly non-coated γFe_2O_3 particles are synthesized by reaction of ferrous dodecyl sulfate with dimethylamine. (4-Amino-1-hydroxybutylidene)bisphosphonic Acid or Alendronate molecules are added in excess to a solution containing non coated nanocrystals. After two hours at room temperature, the precipitate that appears is washed with an acidic solution (HCl 10^{-1} mol.L^{-1}). Free alendronate are isolated from the coated particles thanks to a magnetic field and by

centrifugation. The magnetic γFe$_2$O$_3$@Alendronate are dispersed in water at pH 7.4. The iron concentration is deduced from ultraviolet-visible absorption (Varian Cary 50 Scan UV-Visible spectrophotometer).

The average number of molecules per nanocrystal is deduced with ^{31}P NMR spectroscopy. A range of concentrations of free Alendronate (NMR ^{31}P {1H} (80.9 MHz): 17.076 ppm) solutions added with NaH$_2$PO$_4$ (in capillary, 10^{-1} mol.L^{-1}; NMR ^{31}P {1H} (80.9 MHz): 0 ppm) was prepared for calibration. After chemical decomposition of the magnetic γFe$_2$O$_3$@Alendronate nanocrystals in acidic medium (nitric acid 65%), the ferrous ions are precipitated by addition of sodium hydroxide NaOH (10^{-1} mol.L^{-1}) in order to avoid shifting of the ^{31}P NMR signal. The supernatant is analyzed with ^{31}P NMR and the concentration (number of molecules per nanocrystal) of alendronate into the sample is deduced from this calibration plot.

For the conjugation of Rhodamine B to γFe$_2$O$_3$@Alendronate, 10 mL of ferrofluid γFe$_2$O$_3$@Alendronate in carbonate buffer (n_{Fe} = 10^{-4} mol, n_{ald} = 2,3. 10^{-6} mol) is added to a aqueous (pH=3) mixture containing Rhodamine B (0.239 g, 5.10^{-4} mol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.115 g, 10^{-3} mol) and N-Hydroxysuccinimide (NHS) (0.192 g, 10^{-3} mol). The mixture is stirred for 24 hours at room temperature. The resulting hybrid magneto-fluorescent nanoparticles formed a flocculate, which is dialysed for 4 days. No significant changes in the absorption spectrum of γFe$_2$O$_3$@Alendronate particles are noticed after Rhodamine B grafting. The efficiency of the conjugation is quantitatively evaluated using fluorescence spectroscopy.

The molar ratio of the dye and iron of the magnetic particles was determined after chemical decomposition of the coupling between Rhodamine B and the particles in alkalin medium (pH 12, 24h). The magnetic particles were then separated from the fluorescent supernatant by magnetic decantation. After dilution in water, the dye concentration was deduced from ultraviolet-visible absorption and fluorescence (Rhodamine B, pH=7) and compared to the emission spectrum of an equal molarity of unadsorbed dye in water (Perkin-Elmer LS50B Luminescence spectrofluorometer).

2.2 Liposome Formulation

All liposome formulations were performed using the extrusion technique [14]. 1-Oleoyl-2-Palmitoyl-sn-Glycero-3-Phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Briefly, lipids were dissolved in chloroform and evaporated under nitrogen. The samples placed in a high vacuum during 1 h. The lipid films were rehydrated using either a solution of alendronate ([Al] =1mM), a solution containing γ Fe$_2$O$_3$ @Alendronate ([Fe] = 17.5mM, [Al] =1mM, pH 7.6) by ultrasonic mixing (4 x15 min). The newly formed multilamellar vesicles (MLVs) were extruded 10 times

through polycarbonate membrane filters (Poretics, Livermore, CA, USA) with a decreasing pore size of 400, 200 and 100 nm using an extruder device (Northern Lipids Inc., Vancouver, BC, Canada). Final chromatography through SEPHADEX G-25 gel (GE Healthcare, Buckimghamshire, UK) allowed to get rid of the free compounds and to obtain the final liposomal suspension containing the molecules or nanoparticles of interest.

The liposomal suspension is analyzed with ^{31}P NMR and the average concentration of Alendronate into liposomes is deduced from a calibration plot. The amount of γFe$_2$O$_3$@Alendronate nanocrystals encapsulated into liposomes was determined with UV-Vis spectroscopy (iron concentration).

Size and ζ-potential measurements were achieved by dynamic light scattering and by electrophoretic mobility using a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK).

γFe$_2$O$_3$@Alendronate-Rhodamine and Rhodamine B (Sigma, St. Louis, Mo) were encapsulated with the same procedure described previously and used only for fluorescence imaging and flow cytometry studies. For liposomes containing free Rhodamine or γFe$_2$O$_3$@Alendronate-Rhodamine, a fluorescence study was performed on a Perkin-Elmer LS50B Luminescence spectrofluorometer. A range of concentrations of free Rhodamine B in water and of γFe$_2$O$_3$@Alendronate-Rhodamine were prepared for calibration. The emission spectrum of the fluorescent liposomes is analysed before and after there destruction with Triton in order to evaluate the quenching of fluorescence by liposomes. The concentration of Rhodamine B and of magneto-fluorecsent nanoparticles entrapped into liposomes are deduced from the calibration plots.

2.3 Cell Culture

Brain carcinoma (U87-MG) and epiderm carcinoma (A431). cells were obtained from the American Type Culture Collection. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml streptomycin (all obtained from Life Technologies Inc.), at 37°C in a 5 % CO$_2$ humidified atmosphere. All in vitro cell experiments were carried out at 37°C in a 5 % CO$_2$ incubator.

2.4 Cytotoxicity

Cell viability was evaluated using the MTT microculture tetrazolium assay [15] based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, Mo) into purple formazan crystals. Cells were seeded at a density of 10^4 cells per well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in completed culture medium for 24 h. Then, for A431 cells,

medium was removed and replaced by 2% FCS-medium containing increasing concentrations of alendronate from 100 µM to 1,6 µM and varying concentration of liposomal suspension containing alendronate or γFe_2O_3@Alendronate from 40 µM to 0,31 µM. After 48 h incubation with or without magnet, cells were washed with phosphate buffered saline (PBS, Life Technologies) and incubated with 0.1 ml of MTT (2 mg/ml, Sigma-Aldrich) for additional 4 h at 37°C. The insoluble product was then dissolved by addition of 100 µl of DMSO (Sigma-Aldrich). The absorbance corresponding to the solubilized formazan pellet (which reflects the relative viable cell number) was measured at 540 nm using a Labsystems Multiskan MS microplate reader. The measurement was done in 2 times; the one PBS washed cells and DMSO solubilized formazan pellet. Dose-response curves were obtained for all suspensions with or without magnet, allowing the determination of EC_{50} values, which refer to the concentration inducing a response halfway between the baseline and the maximum plateaued.

2.5 Flow Cytometry

A431 Cells were seeded on Petri dishes (Ø 30 mm, density 50 000 cells per Petri dish), grown for 24 h and treated for 1h: i) with γFe_2O_3@Alendronate - Rhodamine B liposomal suspensions, ii) with γ-Fe_2O_3@Alendronate-Rhodamine B in solutions at the same alendronate and Rhodamine B concentrations than i) and iii) with Rhodamine B liposomal suspensions. This treatment was carried out using magnet or not. Cells were washed twice in PBS. Treated and untreated cells were harvested by trypsinization (500 µl Trypsin-EDTA). Cell suspensions were analyzed with a Beckton Dickinson FACSCalibur 3C (argon laser wavelength at 488 nm) flow cytometer. Ten thousand cells per sample were measured for forward-angle light scattering (FSC) and fluorescence.

3   RESULTS AND DISCUSSION

In order to evaluate the efficiency of various galenic formulation to deliver alendronate on living cells, two human cancer cell lines were chosen: a brain carcinoma (U87-MG) and an epiderm carcinoma (A431). Both adherent cell lines were treated either with free alendronate or, either with γFe_2O_3 nanoparticles grafted with alendronate on their surface (γFe_2O_3@alendronate) or these γFe_2O_3@alendronate entrapped into liposomes. Moreover, when using γFe_2O_3 nanoparticles, cells were treated with and without the presence of a magnet. Alendronate concentration was always measured by ^{31}P NMR. The bilayer lipid composition of the liposomes was plain DOPC. Tables 1 and 2 summarize the results, indicating the maximum of growth inhibition and the EC_{50} values, obtained on the two cells lines, U87-MG and A431 respectively.

	Inhibition Max (%)	(µM)
Free Alendronate	41 ± 8	18 ± 4
Liposomal Alendronate	79 ± 6	2 ± 1
γFe_2O_3@alendronate (no magnet)	42 ± 8	17 ± 4
γFe_2O_3@alendronate (with magnet)	70 ± 10	14 ± 2
Liposomal γFe_2O_3@alendronate (no magnet)	100 ± 2	5 ± 1
Liposomal γFe_2O_3@alendronate (with magnet)	100 ± 1	8 ± 1

Table 1: Treatment of U87-MG cells with various alendronate formulations during 48h.

On U87-MG cells, the liposomal formulations including γFe_2O_3@alendronate nanoparticles gave the best cell growth inhibition. The presence of a magnet increase inhibitory effect using
 SPIONs but was not mandatory for liposomal γFe_2O_3@alendronate nanoparticles.

Treatment	Growth Inhibition Max (%)	EC_{50} (µM)
Free Alendronate	80 ± 10	1 ± 1
Liposomal Alendronate	99 ± 10	1 ± 1
γFe_2O_3@alendronate (no magnet)	93 ± 5	4 ± 2
γFe_2O_3@alendronate (with magnet)	100 ± 5	1 ± 3
Liposomal γFe_2O_3@alendronate (no magnet)	99 ± 2	1 ± 2
Liposomal γFe_2O_3@alendronate (with magnet)	101 ± 2	2 ± 1

Table 2: Treatment of A431 cells with various alendronate formulations during 48h.

On A431 cells, alendronate had already a very good efficiency by itself (80 % of cell growth inhibition). Any other formulation (liposomal or SPION) would just increase up to 100% the maximum rate with no significant change on the EC_{50} value.

However, in an attempt to differentiate the internalization of the different alendronate formulations on this cell line, flow cytometry measurements were carried out; using rhodamine as a fluorescent co-labelling. Figure 1 shows a significant difference in the amount of fluorophore effectively internalized.

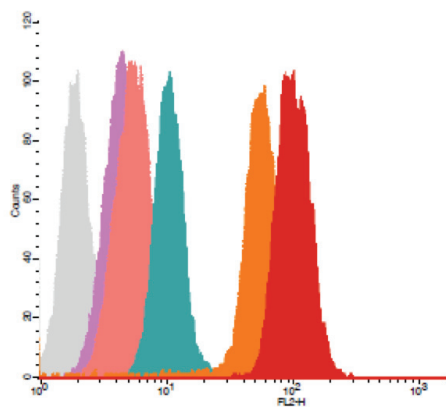| Treatment	Growth	EC_{50}

Figure 1: Alendronate cell uptake measurements by flow cytometry on A431 cells. Rhodamine was used as fluorescent co-label. From left to right: control cell, γFe_2O_3@alendronate-rhodamine (no magnet), γFe_2O_3@alendronate-rhodamine (with magnet), rhodamine-conatining liposome, liposomal γFe_2O_3@alendronate - rhodamine (no magnet), liposomal γFe_2O_3@alendronate-rhodamine (with magnet). Incubation time: 1h at 37°C in culture medium.

It appears that, even if the 100% cell growth inhibition is obtained regardless the formulation used on A431 cells, the maximum of internalization is obtained with liposomal γFe_2O_3@alendronate in the presence of a magnet. Since this formulation gave also the maximum efficiency rate on the other cell line, U87-MG, it should be very promising and be tested on other cell lines and *in vivo*.

4 CONCLUSION

In this communication, we compare the *in vitro* anticancer drug, alendronate delivery on two human cancer cell lines, A431 and U87-MG using various strategies: encapsulation of alendronate into liposome, surface passivation of γFe_2O_3 nanoparticles with alendronate and γFe_2O_3@alendronate entrapped into liposomes. On both cell lines, an effective 100% cytotoxic effect could only be achieved with alendronate grafted-SPIO entrapped into liposomes and within a magnetic field.

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