PEGylated Versus Non-PEGylated γFe₂O₃@Alendronate Nanoparticles

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Abstract

Thanks to their magnetic properties, superparamagnetic iron oxide nanoparticles are considered as a good delivery vehicle after grafting a therapeutic drug on their surface. For additional "stealth" characteristics, PEGylation of surfaces is necessary. The presence of PEG chains divert nanoparticles from their preferred target, the liver macrophages and increased the particle time circulation.

In this work, PEG chain is added to an anticancer drug Alendronate. This molecule is grafted on iron oxide nanoparticle surface in one step surface functionalization method. The in vitro cytotoxic efficiency of γ-Fe₂O₃-Alendronate-PEG nanocrystals is compared with that of free Alendronate, Alendronate-PEG and γ-Fe₂O₃-Alendronate nanocrystals.

Keywords: Magnetic nanoparticle; Alendronate; Anticancer drug; Polyethylene glycol; Nanocarriers

Introduction

In previous work, we have designed new antitumor magnetic nanoparticles by coating iron oxide nanoparticles with a clinically relevant antitumor agent: alendronate [1]. We show that these nanoparticles have both MRI contrast agent and anti-cancer properties. This nanoparticle functionalization spectacularly improved Alendronate cell penetration and its antitumor effect. This nanoparticle functionalization spectacularly improved nanoparticles have both MRI contrast agent and anti-cancer properties.

This new nanoplatform is characterized by various physicochemical methods and the cytotoxicity of the surface modified nanoparticles has been assessed on two cell line models (Human umbilical vein endothelial cells as human normal cells and MDAMB-231 breast human as cancer cell line) using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A comparison between pegylated and non pegylated nanoplatforms is presented.

Materials and Methods

Synthesis of γFe₂O₃@alendronate and γFe₂O₃@alendronate-PEG nanocrystals

Synthesis of uncoated γFe₂O₃ particles was already described [1,3]. A solution of dimethylamine 40% in water ((CH₃)₂NH, 10.5 mL) is added for proteolytic enzymes Decreased accessibility for proteolytic enzymes and antibodies

Figure 1: Schematic representation of the γFe₂O₃@alendronate-PEG nanoplatform nanoparticle.

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to an aqueous micellar solution of ferrous dodecyl sulfate (Fe(DS)₃⁻) (0.61 g, 10⁻³mol). The solution is stirred vigorously for 2 h at 28.5°C and the resulting precipitate of uncoated nanocrystals was isolated from the supernatant at pH=7 by magnetic separation and washed with an acidic solution (HCl 10⁻¹ mol.L⁻¹). The average size of the particle is 10 nm in diameter (see TEM micrograph and size distribution SI). A solution of alendronate or alendronate-PEG molecules (n=10⁻⁴mol in 30 mL of water) was added [3,17,18] to the colloidal suspension. The mixture is stirred for two hours at room temperature. The precipitate is washed with an acidic solution (HCl 10⁻¹ mol.L⁻¹). Free alendronate were isolated from the coated particles thanks to a magnetic field and by centrifugation (3000T/min). Free alendronate-PEG molecules were removed by dialysis. The magnetic nanocrystals coated with alendronate or alendronate-PEG molecules are dispersed in water. The pH was increased to 7.4 by addition of sodium hydroxide (NaOH 10⁻¹ mol.L⁻¹). Iron concentration was deduced from ultraviolet-visible absorption.

**Nanoparticles characterization**

**Transmission electron microscopy (TEM):** The average nanoparticle size was determined with TEM. Samples were prepared by drying a drop of a dilute aqueous solution of γFe₃O₄ nanocrystals onto a carbon coated copper grid. TEM analysis was then carried out on a Philips CM10.

**UV-Visible spectrophotometry:** The iron concentration of the various samples was deduced from UV-visible absorbance spectroscopy experiments (Varian Cary 50 Scan UV-Visible spectrophotometer). Beer-Lambert law was used at 480 nm.

**Infrared spectroscopy (IR):** In order to qualitatively characterize the binding of molecules onto the surface of the γFe₃O₄ nanocrystals via phosphonate groups, IR analysis was performed using a Thermo Electron Corporation Nicolet 380 IR spectrometer.

**Nuclear magnetic resonance (NMR) spectroscopy:** The average number of molecules per nanocrystal is deduced with ³¹P NMR spectroscopy [1,17] using a Varian Gemini spectrometer (200 MHz) and scanned in the range of 0-20 ppm. A range of concentrations of free Alendronate (NMR 3¹P [1H] (80.9 MHz): 17.076 ppm) and free Alendronate-PEG (NMR 3¹P [1H] (80.9 MHz): 17.2 ppm) solutions added with an internal reference NaH₂PO₄ (in capillary, 10⁻¹mol.L⁻¹; NMR 3¹P [1H] (80.9 MHz): 0 ppm) was prepared for calibration. After chemical decomposition of the magnetic γFe₃O₄@alendronate and γFe₃O₄@alendronate-PEG nanocrystals in acidic medium (nitric acid 65%), the ferrous ions were precipitated by addition of sodium hydroxide NaOH (10⁻¹ mol.L⁻¹) in order to avoid shifting of the ³¹P NMR signal. The supernatant containing the free molecules of bisphosphonates was then analyzed with ³¹P NMR (in capillary[NaH₂PO₄] = 10⁻¹ mol.L⁻¹) and the concentration (number of molecules per nanocrystal) of alendronate and alendronate-PEG into the sample was deduced from this calibration plot.

**Dynamic light scattering (DLS):** DLS measurements were achieved using a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK) giving size and ζ potential evaluations. Diluted ferrofluid ([Fe = 5.10⁻³mol.L⁻¹] at pH = 7.4 were used for the measurements of the hydrodynamic diameter and zeta potential, with Nano-ZS device.

**In vitro studies**

**Cell lines and culture:** Human umbilical vein endothelial cells (HUVEC) from PromoCell (Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium supplemented with supplements and growth factors containing hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamycin/ amphotericin-B. Carcinoma cells line (MDA-MB-231) from American Type Culture Collection were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml streptomycin (all obtained from Life Technologies Inc.), at 37 °C in a 5% CO₂ humidified atmosphere. All in vitro cell experiments were carried out at 37°C in a 5% CO₂ incubator.

**In vitro cytotoxicity assay**

Cell viability was evaluated using the MTT microculture tetrazolium assay 26 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 104 cells per well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in completed culture medium for 24 h. Then, for MDA-MB-231, and HUVEC cells, medium was removed and replaced with medium containing increasing concentrations of different formulation of alendronate and alendronate-PEG from 100 μM to 1.6 μM. After 48 h incubation, 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-105 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 performed on DMSO solubilized formazan pellet using PBS washed cells as blank control. Dose-response curves were obtained for all suspensions, allowing the determination of IC₅₀ values, half maximal inhibitory concentration. It is a characteristic measure of the effectiveness of a compound in inhibiting biological or biochemical function. All in vitro experiments had been done in triplicate.
Results and Discussion

Synthesis of a new bisphosphonate: Alendronate-PEG

The Alendronate-PEG (5), is obtained by covalently coupling alendronate molecule (4-Amino-1-hydroxy-1-phosphonobutyl) phosphonic Acid) with polyethylene glycol 200 (1) through a carbamate linkage [19] (Figure 3). The synthesis procedure of precursor molecules and chemical characterization is described in supporting information. The synthesis is performed in four steps. Firstly, one hydroxyl functions of PEG (1) is protected by a benzyl protecting group reacting one eq. of sodium hydride (NaH) with one eq. of PEG 1. The resulting benzylalcohol 2 is activated in the presence of 1.1 eq. of CDI (1,1 '-carbonyldiimidazole) to give the imidazol derivative which can undergo nucleophilic displacement reaction in the presence of alendronate in basic conditions 3. The addition of alendronate amino form allows the carbamate bond formation and the coupling product 4 was obtained in 80% yield. In the last step, deprotection of benzyl group of Alendronate- PEG 4 was carried out by hydrogenolysis. The Alendronate-PEG 5 was obtained in 45% overall yield.

Nanoparticle characterization

Stability and size measurements: Figure 4 shows a TEM photograph and size distribution of nanoparticles after grafting of (A) Alendronate and (B) Alendronate-PEG molecules on nanoparticle surface. In both cases, size distribution are quasi-homogeneous giving a mean diameter of 10 nm and a polydispersity index 0.2 (log normal distribution) as well as uncoated particles [1,3]. The particles are relatively spherical in shape and more or less agglomerated.

The colloidal behavior of γFe₂O₃@alendronate and γFe₂O₃@alendronate-PEG nanocrystals was determined by dynamic laser light scattering (DLS) (Figure 5). Surface charge is a major determinant of solution stability, susceptibility to aggregation and precipitation problems, as well as of protein and cell-surface binding in vivo [20,21]. Considering surface functionalization with alendronate molecules, ferrofluid are stable between 4 to 12 pH range. In this pH domain stability, nanoparticles exhibited an increasing negative zeta potential. The negative surface charge has to be considered in the light of alendronate charges: indeed, these molecules are highly charged, with five pKa values (≈0.8, 2.2, 6.3, 10. P-OH/PO₃⁻, and 12.2 NH₃⁺/NH₂). HMBPs coordinate to iron surfaces via two Fe-O-P bonds (corresponding to pKa₁ and pKa₂), and therefore at pH 7.4, the negative charge is due to the third deprotonated hydroxyl group. Hence, coating with alendronate made the solution anionic. This is well illustrated by

![Figure 3: Synthetic scheme of Alendronate-PEG synthesis.](image-url)
the zeta potential for bare and alendronate-coated nanoparticles as a function of pH (Figure 5). The surface charge was dependent on the coating. The isoelectric point (IEP) of γFe₃O₄@alendronate was about 4.7, and was thus shifted to acidic pH values relative to that for bare nanoparticles. The efficiency of the coating was thereby confirmed by the displacement of the isoelectric point of the nanoparticles as well as by the zeta potential as a function of pH. The mean crystalline core is 10 nm in diameter but at pH=7 the hydrodynamic size is around 32 nm suggesting few aggregates due to a lack of charge at this pH [1,17].

With Alendronate-PEG molecules as surface coating agent, the stability domain extend between 2 to 12 (Figure 6). Moreover, the zeta potential (Figure 5A black curve) as well as hydrodynamic size (Figure 5 B black curve) of γFe₃O₄@alendronate-PEG nanocrystals are nearly constants, 2 mV and 15 nm respectively. The quasi neutral charge could be due to hydroxyl terminal end group of PEG (pKa =16) whereas the PEG chain length favors steric repulsion between particles, reducing aggregation state. Hence, the efficiency of the coating was thereby confirmed by the zeta potential behavior as a function of pH.

**Average number of molecules per nanoparticle: NMR Quantification:** In order to perform a comparative biological study between the two nanoplatforms, it is necessary to estimate the average number of grafted molecules on the nanoparticle surface. For this, we used the method described in materials and methods (2.3.4). Briefly, after chemical decomposition of the magnetic γFe₃O₄ nanocrystals in acidic medium, the supernatant containing free molecules is analyzed using the method described in materials and methods (2.3.4). Briefly, after chemical decomposition of the magnetic γFe₃O₄ nanocrystals in acidic medium, the supernatant containing free molecules is analyzed by amine terminal end group with alendronate compared to hydroxyl end group of alendronate-PEG.

![Figure 5: (A) pH-dependent zeta potential and pH-dependent hydrodynamic size curves for γFe₃O₄@alendronate (red) and γFe₃O₄@alendronate-PEG (black) nanocrystals.](image)

**In vitro cytotoxicity measurement:** The anti-cancerous activity of free molecules and coated nanocrystals was evaluated in vitro using HUVEC (Human umbilical vein endothelial cells) as human normal cells and MDA-MB-231 breast human cancer cell line (Figure 8).

Dose–response curves were obtained for all suspensions allowing the determination of IC₅₀ values, which refer to the concentration inducing 50% growth inhibition. Table 2 summarizes all the cytotoxicity results.

From the results in Table 2 and the figure 8, it could be emphasized that free molecules as well as coated nanocrystals do not affect the viability of normal cells (HUVECs). Moreover, it is clear that cytotoxicity of alendronate on MDA-MB-231 cells is reduced by addition of PEG chain. In particular, the IC₅₀ value increases around 3.5 times. The differences in the responses of the cell lines to alendronate may be due to differences in membrane permeability or sensitivity to its mechanism of action. Cancer cells are more permeable than normal cells, which explains the lack of efficiency of the alendronate against HUVEC cells. Grafting the molecules (alendronate and alendronate-PEG) on γ-Fe₂O₃ nanoparticles improves the efficiency. Indeed, the IC₅₀ is reduced around 3-4 and 2.5 for alendronate and alendronate-PEG respectively. It is well known that cellular uptake of nanoparticles depends highly upon size and charge of the nanoparticles [22,23]. For example it has been shown with quantum dots (QD) that nearly neutral nanoparticle with hydroxyl (-OH) surface functionalization, exhibit greatly reduced non-specific internalization⁶, when compared with the carboxyl or amino counterparts, so charge is of a definitive importance to understand the internalization characteristics. While the uptake

![Figure 4: TEM micrograph and size distribution of γFe₃O₄@alendronate (A and C) and γFe₃O₄@alendronate-PEG (B and D) nanocrystals.](image)
mechanisms of nanoparticles into cells is defined as non-specific, these pathways are initiated by surface receptors, either through direct interactions between the charged nanoparticle and the receptor or via proteins adsorbed on the nanoparticles surface. It has been shown that the presence of PEG layer on the nanoparticle surface induced steric hindrance by physically separating the surface from that of the cell (steric hindrance), hence reducing nanoparticle adherence to the cell membrane and leading to reduced internalization. PEG also acts efficiently to reduce protein adsorption onto the nanoparticle surface [22-25]. This property has been shown to prevent phagocytosis, as well as reducing the binding of nanoparticles to the cell membrane, and hence lowering internalization. As can be seen, γFe₃O₄@alendronate-PEG nanoparticles had zeta potentials closer to zero compared to negatively γFe₃O₄@alendronate- charge surface. The difference in the IC₅₀ values between pegylated and non pegylated nanoparticles could be attributed to charge effect and reduced cellular uptake with γFe₃O₄@ alendronate-PEG nanoparticles.

Conclusion

In this paper, alendronate-PEG-modified iron oxide nanoparticles about 15 nm in hydrodynamical size have been prepared using one step surface functionalization method and characterized by various physico-chemical means. The colloidal solution of nanoparticles presents high stability on large pH range. In vitro studies show that these nanoparticles exhibit non toxicity on HUVEC cells and anti-tumoral potency on MDAMB-231 breast human cancer cells.

It can be expected that the addition of PEG chains to γFe₃O₄@alendronate nanoplateform could increase their particle time circulation, reduce immunogenicity, and also promotes their accumulation in tumors due to enhanced permeability and retention effect. The terminal OH group of PEG may be selectively oxidized to functionalize PEG with various terminal end groups such as targeting biomolecules.
or fluorofores [26]. The effectiveness of this new antitumoral nanoplatform has to be effectively evaluated in vivo.

Supporting information

Synthesis of (4-Amino-1-hydroxy-1-phosphonobutyl) phosphonic acid (Alendronate): Alendronate was synthesized according to the general procedure [1] for Aminobisphosphonate molecules (BPs) and characterized by ¹H and ³¹P NMR. Briefly, BP was prepared from the corresponding carboxylic acid precursor 4-aminobutyric acid as followed. Carboxylic acid (150 mmol) and H₃PO₄ (150 mmol) were introduced in a three-necked round-bottom flask under inert atmosphere followed by 30 ml of methanesulonic acid. After heating at 65°C for 1 h, PCI₃ (40 mmol) was slowly added and the reaction was allowed to proceed overnight at 65°C. The resulting yellow viscous reaction mixture was cooled to room temperature, quenched with 500 ml of ice-cold water. The pH was adjusted to 4.3 with a NaOH aqueous solution (0.5 M) and the obtained white precipitate was collected by filtration. This solid was washed five times with a mixture of methanol/water (95:5), dialyzed for 3 days and freeze-dried to finally obtain Alendronate as a sodium salt.

Yield: 82%

I.R. (cm⁻¹) : (KBr): 1540, 1172, 1052 cm⁻¹
RMN ¹H (80.9 MHz, D₂O): 18.5
RMN ³¹P (161.9 MHz, D₂O): 161.9

Stock solution was prepared at 10 mM in deionized water. Dilutions of the stock solution were conducted with Eagle's medium, and the highest tested concentration corresponded to 1 mM.

Synthesis of 2-{2-[2-(2-Benzoyloxy-ethoxy)-ethoxy]-ethoxy}-ethanol 2: Sodium hydride (50 mmol, 1 eq.) was introduced in a three-necked flask, in 50 mL of dry THF with stirring under inert atmosphere. Polyethylene glycol 200 (50 mmol, 1 eq.) was added drop wise and at 0°C. The reaction mixture thus obtained was stirred and refluxed for 1h 30 in absence of light. 8.55 g of benzyl bromide (50 mmol, 1 eq.) was then added drop wise at room temperature. The evolution of the reaction was monitored by TLC with dichloromethane-ethanol (9/1) as eluent. Once completed, the reaction mixture NaBr salts was filtered. The recovered filtrate was then dried over anhydrous MgSO₄, filtrated and evaporated under reduced pressure. A brown oil was obtained and purified by chromatography column on silica using dichloromethane - ethanol (95/5) as eluent.

Yield: 70%

I.R. (cm⁻¹) : 940, 1100, 1249, 1351, 1453, 2867, 3453
RMN ¹H (200 MHz, CDCl₃): 2.54 (s, 2H), 3.66 (m, 14H), 4.56 (s, 2H), 7.32 (s, 5H)

Synthesis of Imidazole-1-carboxylique 2-{2-[2-(benzyloxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester 3: Carboximidazole (CDI) (30.8 mmol, 1.1 eq.) was dissolved, under inert atmosphere, in 40 mL of previously distilled acetonitrile. After several minutes of stirring, alcohol 2 (28 mmol, 1 eq.) was added dropwise at 0°C, the reaction mixture was placed in an ice bath. The solution was yellow. After 15 minutes of stirring, the mixture was cooled to room temperature; the reaction progress was followed by TLC with dichloromethane-ethanol (9/1) as eluent. Once completed, the reaction mixture was then filtered. The filtrate recovered was then dried over anhydrous MgSO₄ and evaporated under reduced pressure. A brown oil was recovered obtain. The resulting desired activated alcohol was immediately engaged in the following reaction without further purification.

I.R. (cm⁻¹) : 754, 1099, 1270, 1763, 2869
RMN ¹H (200 MHz, CDCl₃): 3.60 (m, 12H), 4.50 (s, 2H), 6.98 (s, 2H), 7.28 (s, 5H), 7.65 (s, 1H)

Synthesis of [4-2-[2-(2-Benzoyloxy-ethoxy)-ethoxy]-ethoxy]carboxyaminolino]-1-hydroxy-1-phosphono-butyl]-phosphonic acid 4: In a 500 ml flask, protected from light, previously obtained activated alcohol 3 (19.14 mmol, 2 eq.) was dissolved in 20 mL of dry dichloromethane at room temperature. After several minutes of stirring, alendronate (9.57 mmol, 1 eq.) dissolved in 20 mL of water was added drop wise. The solution of alendronate was previously set to pH 12. The evolution of the reaction was monitored by ³¹P NMR. Once completed, the two phases were separated. The aqueous phase was evaporated. The white obtained solid was precipitated in 50 mL of acetone. It was purified by reverse phase chromatography column using water - methanol (95/5) as eluent.

Yield: 80%

RMN ¹H (200 MHz, D₂O): 1.9 (m, 4H), 3.20 (t, 2H), 3.75 (m, 14H), 4.19 (s, 2H), 4.66 (s, 2H), 7.31 (s, 5H)
RMN ³¹P (161.9 MHz, D₂O): 18.90 (s)
MS: (C₁₈H₂₆NO₁₅P₂) m/z: [M + Na]+ = 582.16; [M + 2Na - H]+ = 604.14; [M + 3Na - 2H]+ = 626.11; calc: 559.

Synthesis of [1-Hydroxy-4-[2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy]carboxyaminolino]-1-phosphono-butyl]-phosphonic acid 5: Protected Alendronate-PEG 4 was dissolved in 5.4 mL of deionised water. 0.06 g of palladium on charcoal 10% (30% by weight) was added with stirring. Air was replaced by hydrogen. After 24 hours stirring, the reaction was complete. The solution was centrifuged and the supernatant was lyophilized to obtain a white powder.

Yield: 80%

RMN ¹H (200 MHz, D₂O): 1.87 (m, 2H), 2.10 (m, 2H), 3.16 (t , 2H)
RMN ³¹P (161.9 MHz, D₂O): 3.72 (m, 14H), 4.21 (s, 2H)
I.R. (cm⁻¹) : 541, 1139, 1268, 1701, 2923
RMN ³¹P (161.9 MHz, D₂O): 18.13 (s)
MS: (C₁₈H₂₆NO₁₅P₂) m/z: [M + H]⁺ = 470.42; calc: 469.

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