Cytokine adsorption/release on uniform magnetic nanoparticles for localized drug delivery

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1. Introduction

Cancer immunotherapy is at present an active field of biomedical research given its relevance in the design of new anti-tumor devices. Treatments based on activating an anti-tumor immune response would not only allow removal of the primary tumor, but could also provide sufficient protection to eliminate neoplastic cells that spread to form distal metastases [12]. There is considerable recent pre-clinical and clinical evidence showing that manipulation of the innate and adaptive immune responses to neoplastic cells can control development of tumors, leading to their elimination [3–5]. This has led to the concept that immunotherapy might be a central element in the development of anti-cancer therapies, in combination with surgery as well as chemotherapeutic agents [6].

Studies of the anti-tumor immune response indicate that the T helper type 1 (Th1) lymphocyte-mediated response is much more effective than that mediated by Th2 lymphocytes [7]. Due to their increased cytotoxic activity, Th1 cells have a greater capacity than Th2 cells to eradicate tumors in murine models [7]. Type Th1 responses are characterized by the production of interferon gamma (IFN-γ) on negatively charged magnetic nanoparticles prepared by three different methods, including coprecipitation, decomposition in organic media, and laser pyrolysis. To facilitate IFN-γ adsorption, magnetic nanoparticles were surface modified by distinct molecules to achieve high negative charge at pH 7, maintaining small aggregate size and stability in biological media. We analyzed carboxylate-based coatings and studied the colloidal properties of the resulting dispersions. Finally, we incubated the magnetic dispersions with IFN-γ and determined optimal conditions for protein adsorption onto the particles, as well as the release capacity at different pH and as a function of time. Particles prepared by decomposition in organic media and further modified with dimercaptosuccinic acid showed the most efficient adsorption/release capacity. IFN-γ adsorbed on these nanoparticles would allow concentration of this protein or other biomolecules at specific sites for treatment of cancer or other diseases.

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although this approach poses serious problems for clinical application [14]. Synthetic polymers and microspheres generated with gelatin and chondroitin sulfate have also been tested as controlled cytokine release systems [15–17], although to be effective, the microspheres must reach the tumor at the appropriate concentration. Nanotechnology provides valuable new tools that would allow controlled local release of cytokines. One of the most promising methods for delivery of drugs to their points of action is by binding them to magnetic particles that are then targeted using an external magnetic field. The first trial of magnetism to attract a drug to the site of action used intravenously injected epirubicin-coated magnetic particles. After injection, a magnetic field was applied as close to the tumor as possible for 45 min, resulting in efficient drug administration to the site [18].

To enable a more extensive study of the IFN-γ effect on tumor progression by modulating the Th1 immune response in the tumor microenvironment, it was necessary to optimize a magnetic nanoparticle-based system for IFN-γ delivery. Three different methods were used to produce uniform magnetic nanoparticles (polydispersity degree <20%) with different crystallinity and surface characteristics. One was a conventional method of coprecipitation of Fe salts in water, widely used for commercial products and for previous studies of biomolecule immobilization. Another was a new method based on the decomposition of an organic precursor in organic media, which assures formation of uniform, highly crystalline particles; finally, we used the laser pyrolysis technique, a unique method for producing dry powders in a single step starting from a gaseous precursor [19,20]. We analyzed the adsorption/release of murine interferon gamma (IFN-γ) onto these iron oxide nanoparticles with various coatings. Particle size and crystal structure, as well as the coating, affect the magnetic properties of the material [19] and are therefore predicted to have an effect on its adsorption/release capacity.

2. Materials and methods

2.1. Nanoparticle synthesis

Three samples were synthesized using coprecipitation in alkaline media of iron salts (sample C), as well as thermal decomposition in an organic solvent (sample O) or by laser pyrolysis (sample L) of a metal-organic precursor. The particle surface was appropriately modified in each case to achieve high negative charge, small aggregate size and stability in biological media. Sample C consists of dextran-coated magnetite nanoparticles synthesized in a single step by coprecipitation of iron (II) and (III) salts in a basic ammonium solution in the presence of dextran [21]. Samples O and L were synthesized in two-step processes. For sample O (DMSA-coated), hydrophobic magnetite nanoparticles were synthesized by decomposition of iron acetylacetonate in phenyl ether [22,23]; in a second step, the nanoparticles were surface modified with DMSA to remove the oleic acid and render a negative charge at pH 7 [24,25]. Sample L was synthesized by laser pyrolysis of an iron pentacarbonyl aerosol following a reported procedure [26,27]. Particles were then boiled in nitric acid and iron nitrate to increase crystallinity [28], and finally coated with one of three molecules, phosphonoacetic acid (sample LP), citric acid (sample LC) or carboxydextran (sample LCD) [29,30]. A control sample consisting of iron oxide particles with a positive surface charge was prepared by coprecipitation in the presence of aminodextran (control sample).

2.2. Structural and colloidal characterization

Particle size, shape and degree of polydispersity were analyzed by transmission electron microscopy (TEM) using a 200-KeV JEOI-2000 FXII microscope. TEM samples were prepared by placing one drop of a dilute suspension of magnetite nanoparticles on a carbon-coated copper grid and allowing the solvent to evaporate at room temperature. Average particle size (DTEM) and distribution were evaluated by measuring the largest internal dimension of at least 300 particles. Data were fitted with a log-normal distribution function and the logarithmic standard deviation was obtained for the samples. The standard deviation was calculated from the logarithmic standard deviation as described [31].

The phase of the iron oxide particles was identified by powder X-ray diffraction. The X-ray patterns were collected between 20–70 (2θ) degrees in a Bruker D8 Advance diffractometer with CuKα radiation. Crystal size (DXRD) was calculated from the broadening of the (311) reflection of the spinel structure following standard procedures. Hydrodynamic size and evolution of the zeta potential versus pH was determined using a Zetasizer Nano-ZS device (Malvern Instruments). Hydrodynamic size was measured from a dilute suspension of the sample in water at pH 7 in a standard cuvette. Zeta (Z)-potential was also measured from dilute samples in water in a Zeta Potential cuvette with 0.01 M KNO₃ as support electrolyte and at different pH values.

Thermogravimetric analysis (TG) of samples was carried out in a Seiko TG/ATD 320 U, SSC 5200 (Seiko Instruments) to evaluate material weight loss during heat treatment. For this analysis, a heating rate of 10 °C/min in air atmosphere from room temperature to 700 °C was applied to the sample. Fourier transform infrared spectra (IR) of the iron oxide nanoparticles were recorded between 3600 and 400 cm⁻¹ in a Nicolet 20 SX FTIR to confirm the iron oxide phase, the nature of the coating and its bonding to the surface. Samples were prepared by diluting iron oxide powder in KBr at 2% by weight and pressing into a pellet.

Iron concentration of the samples was analyzed in a plasma emission spectrometer (ICP; Perkin Elmer Optima 2100 DV) after sample digestion in nitric and hydrochloric acids.

2.3. Cytokine adsorption/release

For analysis of the adsorption process, nanoparticles were added in excess to the medium with 2 ng/ml murine IFN-γ (Peprotech) and incubated (2 h, room temperature (RT), with shaking). The recombinant murine IFN-γ is a homodimeric protein. Each subunit has 134 amino acids and a molecular weight of 15.65 kDa, and the chemical formula is C₁₀₉H₁₅₀N₁₉₅O₂₅S₂. Nanoparticles were separated magnetically from supernatants and the supernatant (unbound IFN-γ) was collected for analysis. Nanoparticles were resuspended in appropriate medium with 1% bovine serum albumin (BSA; Calbiochem), washed (30 min, RT, with shaking) and separated from supernatant (washed IFN-γ). To determine the best conditions for the IFN-γ desorption after nanoparticle resuspension, the pH of the medium was modified between 5.5 and 9 at 0.5 unit intervals, using HCl or NaOH in 0.5% NaCl. Finally nanoparticles were separated magnetically from the suspension and supernatants again collected for analysis (released IFN-γ).

Other experimental parameters were optimized for samples C and O, including magnetic separation time (to avoid interference by remaining magnetic nanoparticles in the medium), ionic strength, adsorption pH, and adsorption and release times. The effect of particle coatings on adsorption/release was then studied under the optimized experimental conditions.

2.3.1. Effect of ionic strength of the medium on IFN-γ adsorption

To test the effect of the ionic strength of the medium, we used magnetic nanoparticles, one with a weak (sample C) and one with a strong negative surface charge (sample O). Both were incubated in excess with 2 ng/ml IFN-γ in phosphate-buffered saline (PBS; Roche) 0.9% NaCl, or 0.5% NaCl (2 h, RT, with shaking), after which nanoparticles were separated using magnets and supernatants were collected for analysis.

2.3.2. Effect of pH of the medium on IFN-γ release

After incubation of dextran (C)-, and DMSA (O)-coated particles in 0.5% NaCl, pH 7 with IFN-γ (1 ng/ml; 2 h, RT, with shaking), particles
were separated magnetically from supernatants (1 ml aliquots, 30 min, RT). Supernatants were collected and nanoparticles resuspended in 0.5% NaCl with 1% BSA, and then washed (30 min, RT, with shaking), after which nanoparticles were re-isolated and supernatants obtained. To release IFN-γ from particles, washed IFN-γ-coated nanoparticles were resuspended in 0.5% NaCl in a pH range from 5.5 to 9 (0.5-unit intervals) and incubated (2 h, RT, with shaking), followed by magnetic isolation of particles and supernatant collection.

2.4. IFN-γ detection

Unbound, washed and released IFN-γ were detected by enzyme-linked immunosorbent assay (ELISA) using the Mouse IFN-γ ELISA Set (BD Biosciences). Some samples were further analyzed by Western blot with a rabbit anti-murine IFN-γ polyclonal antibody (0.2 µg/ml, Peprotech) and a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (0.15 µg/ml, Dako).

2.5. Functional IFN-γ assay after adsorption/release

Murine macrophages were obtained from C57BL/6 mice by peritoneal injection of casein (9% w/v in PBS). After 24 h, macrophages were collected from the peritoneum by washing with ice-cold PBS in sterile conditions. After centrifugation (350 ×g, 5 min) cells were resuspended in complete RPMI medium (10% FCS, l-glutamine, penicillin/streptomycin, Na-pyruvate, non-essential amino acids and β-mercaptoethanol), cultured in 6-well plates (2 × 10⁶ cells/well in 3 ml) and incubated for 20 h in standard cell culture conditions (37 °C, 5% CO₂ in humidified air). Cells were washed with PBS and treated appropriately with 10 ng/ml IFN-γ released from nanoparticles, IFN-γ bound to DMSA-coated nanoparticles or free IFN-γ as positive control; an untreated well was used as a control. After 24 h incubation, treated cells were washed with PBS, collected and stained with anti-CD40-PE and anti-CD11b-FITC (both from BD Pharmingen) antibodies and analyzed by flow cytometry. All procedures using animals were carried out with the approval of the CNB Ethics Committee following EU guidelines.

3. Results and discussion

3.1. Synthesis and characterization of the nanoparticles

Magnetic nanoparticles were prepared by coprecipitation and by decomposition of a metal-organic precursor, either in solution or from an aerosol. These methods were optimized to obtain uniform particles, with a polydispersity degree <20% and thus within the monodispersed range (Table 1); TEM images of the iron oxide nanoparticles are shown in Fig. 1. Particles prepared by decomposition and laser pyrolysis had an average diameter of 4.5 and 10 nm, respectively, and standard deviations in the monodispersed range (~0.20). Iron oxide nanoparticles synthesized by coprecipitation in the presence of dextran (sample C) had a 10 nm diameter and a standard deviation of 35%. The presence of dextran reduces and regulates particle growth, but for sample C, particle size distribution (a consequence of the mechanism of particle formation [19,20]) was still wider than that for the decomposition synthesis methods (samples O, L).

We characterized the nature of the iron oxide nanoparticles and their surface coating by X-ray diffraction and IR spectroscopy (Figs. 2, 3). All diffraction peaks corresponded to spinel structure similar to magnetite or maghemite, and the crystal size calculated from the peak broadening (sample C=10 nm, sample O=5 nm, sample L=9 nm) concurred with mean TEM size (Table 1 and Fig. 2). In the low frequency IR area, two strong absorption bands were assigned at 390 and 600 cm⁻¹ to the Fe–O stretching of iron oxide (Fig. 3). The shape and position of these bands in samples C and O suggest that the majority of iron oxide is magnetite, although some oxidation has taken place and maghemite is also present [32]. The appearance of new shoulders at higher frequency in this IR region indicated that laser pyrolysis samples L and LP consist mainly of...
magnetite rather than magnetite; this is due to the oxidant conditions of the recrystallization process.

In the intermediate IR region, we identified surface coating molecules as well as synthesis-derived impurities, i.e., the sharp band at 1380 cm\(^{-1}\) due to nitrate impurities remaining from acid recrystallization (samples L, LP) (Fig. 3). From 1150 cm\(^{-1}\) to 1000 cm\(^{-1}\), multiple strong absorption bands appear in the C and LP spectra due to the C–O single bond stretching modes of the dextran and phosphonoacetic acid, respectively. Sample LP showed broad absorption bands in this region, with bands at 1050 and 1010 cm\(^{-1}\) corresponding to the split triply degenerate \(\nu_3\) (P–O–) vibration and at 980 cm, corresponding to the \(\nu_1\) (P–O). This assignment is commonly associated with a monodentate bond between particle and phosphonate [33]. The small differences in band intensity and position between this sample and those previously reported are due mainly to pH and to phosphonate concentration.

In the high energy range, the two absorption bands near 3000 cm\(^{-1}\) were due to C–H stretching (anti-symmetric and symmetric). These bands were partially masked for samples C and LP, because of the intense O–H stretching modes of dextran hydroxyl groups and water molecules. At 1630 cm\(^{-1}\), we observed a weak absorption band in all spectra due to adsorbed water. This peak was more intense in sample O because of the overlapping of the DMSA C=O stretching mode.

Thermogravimetric analyses of the powder samples were used to quantitate the coating (Table 1). In the case of the phosphate-coated sample (LP), phosphate is not lost after heating up to 1000 °C; analysis of Fe content was therefore carried out by elemental analysis by ICP. The weight percentage of coating molecules adhered to the nanoparticle surface increased from 14% for dextran-coated sample C to 24% for DMSA-coated sample O and 64% for the carboxydextran-coated sample (LCD). This LCD > O > C coating percentage trend did not correlate as predicted with the increase in particle size (O > C > LCD), indicating that surface nature and negative charge are the principal factors that affect the amount of coating adsorbed on the nanoparticle surface.

3.2. Characterization of the suspensions

All negatively charged samples had similar hydrodynamic sizes (~100 nm), which is essential for comparison of the adsorption/release process between IFN-γ and the coated nanoparticles (Table 1); the amount of IFN-γ bound might otherwise vary as a function of aggregate size, interfering with sample comparison. The hydrodynamic size values were stable for months and sedimentation was not observed.

Nanoparticle dispersion stability depends on charge and surface chemistry, which give rise to both steric and electrostatic repulsion [34,35]. Conventionally, a zeta potential higher than ±30 mV would be considered stable, i.e., the dispersion will resist aggregation [35]. The evolution of the Z-potential vs. pH for samples coated by different molecules is shown in Fig. 4. In the case of DMSA-, phosphonoacetic- and citric acid-coated samples (O, LP and LC), the ionizable carboxylate groups on their surfaces govern the pH-dependent evolution of the Z-potential, whose high values resemble those reported in similar cases [35]. A pure polymer-coated sample, such as dextran sample C, shows lower electrostatic stabilization (lower Z-potential), due to the lack of ionizable surface groups; the steric effect helps to maintain a stable dispersion, independently of the pH. For the carboxydextran-coated sample (LCD), the charged polymer on the surface provides both stability factors. The electrostatic effect is reflected in the Z-potential values, higher than those for sample C and similar to samples LC and LP. The pH dependence of the Z-potential thus showed intermediate variation between sample C and samples LC and LP. Finally, the uncoated pyrolysis sample (L) shows the typical Z-potential values reported for bare iron oxide nanoparticles, which is ~0 at pH 7 [36].

3.3. Optimization of IFN-γ adsorption/release on magnetic nanoparticles

3.3.1. Magnetic particles interfere with IFN-γ detection in ELISA

The presence of a small quantity of magnetic nanoparticles in the medium interfered severely with the IFN-γ detection assay (Fig. 5A), underlining the need to apply magnetic separation for a time period sufficient for complete removal of nanoparticles from the medium. Several experiments indicated an optimal separation time of 30 min for this study, allowing particle removal while avoiding IFN-γ degradation (not shown).

3.3.2. Effect of ionic strength of the medium on adsorption

The ionic strength of the medium was predicted to have a strong negative effect on the adsorption process. As nanoparticle/IFN-γ binding is based on attraction between opposite surface charges, the
ionic cloud that forms around the particle could interfere with adsorption. We observed greater adsorption in low ionic strength medium (Fig. 5B), and thus used a 0.5% NaCl solution in further experiments. The amount of IFN-γ lost in the adsorption process was greater when we used weakly charged nanoparticles, especially in high ionic strength media (Fig. 5B), confirming the electrostatic nature of the nanoparticle:IFN-γ interaction.

### 3.3.3. Effect of pH on IFN-γ release

We subsequently established appropriate pH conditions for IFN-γ release from the nanoparticles. The largest amount of cytokine was released at pH 8 (Fig. 5C). This supported the concept that interaction is due mainly to attraction between opposite surface charges, since the pH for release is near the theoretical isoelectric point (pI) for murine IFN-γ. As predicted, positively charged control nanoparticles adsorbed only negligible amounts of IFN-γ (see below). We observed a second release peak at low pH (5.5), which was not used in the experiments, as it is far from physiological conditions (pH 7.4).

**Fig. 5.** Optimization of IFN-γ adsorption/release from nanoparticles. (A) The presence of nanoparticles in the medium interfered with ELISA assay for IFN-γ detection (O, DMSA-coated; C, dextran-coated; LP, phosphonoacetic acid-coated; LC, citrate-coated; LCD, carboxydextran-coated; Control, aminodextran-coated). (B) ELISA using two types of nanoparticles showed that the amount of IFN-γ lost during the adsorption process was dependent on ionic strength. (C) ELISA analysis of IFN-γ release from nanoparticles as a function of pH. (D) Analysis of IFN-γ release as a function of time. A–D show mean±SD for triplicate samples from a representative experiment of three performed.

**Fig. 6.** IFN-γ adsorption/release from nanoparticles. (A) In ELISA, unbound IFN-γ in binding medium and washes varied according to nanoparticle surface coating. (B) ELISA showing IFN-γ release at pH 8. (C) ELISA showing IFN-γ release from O nanoparticles at RT or at 37 °C. A, B, and C show mean±SD for triplicate samples from a representative experiment of three performed. (D) Western blot detection using sample O and control to analyze IFN-γ adsorption/release. +, commercial IFN-γ; O-IFN-γ, washed IFN-γ-adsorbed sample O nanoparticles; O, unadsorbed sample O nanoparticles; Control-IFN-γ, washed IFN-γ-adsorbed sample control nanoparticles.
3.3.4. Effect of incubation time on IFN-γ release

To determine incubation times that would allow maximum IFN-γ release with minimum degradation, we used IFN-γ bound to samples C and O. Particles were resuspended in 0.5% NaCl, pH 8, and incubated (1, 2, 3, or 20 h, RT with shaking). After magnetic removal of nanoparticles, supernatants were analyzed by ELISA; IFN-γ release was most efficient at 2 h, with no notable increase thereafter (Fig. 5D). The amount of IFN-γ decreased at longer incubation times (3–20 h), probably due to degradation.

3.4. IFN-γ adsorption/release as a function of nanoparticle surface charge

The IFN-γ adsorption/release process was studied for several types of negatively charged magnetic nanoparticles, using positively charged particles (aminodextran-coated) as a control. We measured IFN-γ loss as unbound IFN-γ in binding medium and washes. We found no IFN-γ binding to positively charged nanoparticles, whereas it bound to negatively charged nanoparticles to a greater or lesser extent, depending on the surface coating (Fig. 6A). Attractive electrostatic interaction was thus observed between negatively charged nanoparticles and IFN-γ at pH 7, with DMSA (sample O)- and carboxydextran (LCD)-coated nanoparticles showing the most efficient IFN-γ binding.

We tested all coated nanoparticles in the conditions described above to determine which released the most IFN-γ. Both DMSA (sample O)- and carboxydextran (LCD)-coated nanoparticles released approximately 75% of the initial IFN-γ bound; both IFN-γ binding and release were thus efficient using these nanoparticles (Fig. 6B). Other negatively charged nanoparticles showed lower IFN-γ release values, whereas positively charged aminodextran particles did not release protein, as they were unable to bind it. Considering potential in vivo applications, we tested IFN-γ released by sample O at 37 °C, (standard human body temperature and thus, that of cell culture). Results were similar to those obtained at RT (Fig. 6C).

We also analyzed IFN-γ adsorption/release on DMSA-coated nanoparticles by immunoblotting, using positively charged aminodextran-coated control particles. The presence of nanoparticles did not interfere with IFN-γ detection by Western blot. Results were similar to those obtained in ELISA (Fig. 6D).

3.5. IFN-γ function following adsorption/release from nanoparticles

IFN-γ activates macrophages, leading to increased production and secretion of several cytokines, as well as of certain membrane proteins, including CD40 [37,38]. To determine IFN-γ function, we used nanoparticle-released IFN-γ to activate primary murine macrophages in vitro and analyzed cell CD40 expression by flow cytometry. The nanoparticle adsorption/release process did not modify IFN-γ function, as the percentage of activated macrophages expressing CD40 and mean fluorescence levels were similar for IFN-γ-adsorbed sample O nanoparticles, IFN-γ released from sample O nanoparticles, and for the commercial IFN-γ control (Fig. 7).

4. Conclusions

We successfully prepared and characterized a series of uniform magnetic nanoparticles with different coatings and hydrodynamic sizes around 100 nm. Various synthesis methods were developed and adjusted to meet the requirements for biomedical applications. Changes in synthesis method and particle coating not only altered the crystalline nature of the particles, but also modified their capacity to adsorb and release cytokines. We measured the IFN-γ adsorption/release capacity of the magnetic nanoparticles and evaluated the possibilities for drug delivery in biomedical applications. Particles prepared by decompositon in organic media and further modified with dimercaptosuccinic acid showed the most efficient adsorption/release capacity. This preparation method is one of the most promising routes for the synthesis of nanoparticles with narrow particle size distribution and high crystalline character. IFN-γ released from the particles was functional, as it activated macrophages in vitro. These results support the design of further experiments to test the in vivo potential of these delivery systems for cytokine targeting to a tumor site.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2008.05.028.

References


