Nucleic Acids Research, 1998, Vol. 26, No. 23 **5425–5431**

Highly efficient cellular uptake of *c-myb* antisense oligonucleotides through specifically designed polymeric nanospheres

Luisa Tondelli, Alfredo Ricca[1], Michele Laus[2], Marco Lelli[2] and Gennaro Citro[1,*]

Consiglio Nazionale delle Ricerche, I.Co.C.E.A., Via Gobetti 101-40129 Bologna, Italy, [1]Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Roma, Italy and [2]Dipartimento di Chimica Industriale e dei Materiali, Università di Bologna, Viale Risorgimento 4, 40136 Bologna, Italy

Received July 27, 1998; Revised and Accepted September 30, 1998

ABSTRACT

***c-myb* antisense oligonucleotides (AS ODNs) were reversibly immobilized to a novel polymeric core shell nanosphere and their cellular uptake and inhibitory effect on HL60 leukemia cell proliferation studied. The nanosphere surface was so designed as to directly bind ODNs via ionic interactions and reversibly release them inside the cells. Compared with the cellular uptake of free oligonucleotide, the use of AS ODN (immobilized to the nanospheres) produced a 50-fold increase in the intracellular concentration. Specifically, a single dose of 320 nM of AS ODN immobilized to the nanospheres was capable of inhibiting HL60 cell proliferation with the same degree of efficiency obtained using a 50-fold higher dose of free AS ODN. Flow cytometric experiments with fluoresceinated ODNs showed a temperature-dependent uptake, which was detectable as early as 2 h after the beginning of treatment. The inhibitory effect on cell proliferation was maintained for up to 8 days of culture. Moreover, the level of *c-Myb* protein decreased by 24% after 2 days and by 60% after 4 days of treatment, thus indicating a continuous and sustained release of non-degraded AS ODN from the nanospheres inside the cells.**

INTRODUCTION

Antisense oligonucleotides (AS ODNs) have been successfully employed to specifically inhibit gene expression both *in vitro* and *in vivo* (1–4). As the bioavailability of unmodified ODNs is seriously reduced by their fast degradation in serum and cells induced by ubiquitous exo- and endonucleases, several chemical modifications have been performed to make them more resistant to enzymatic degradation. Phosphorothioate-modified oligodeoxynucleotides (PS ODNs) are the most widely employed analogues due to their greater stability to nucleases while still maintaining their biological activity (5). In addition, their synthesis is easy and can be performed under mild conditions. Approximately 10 PS ODNs are currently being used in human clinical trials as both antiviral (mainly HIV-related diseases) and antitumor (chronic myelogenous leukemia) agents (2). However, contradictory results concerning the actual biological mechanisms of PS ODNs have been reported. Some studies indicate that PS ODNs act by sequence-specific mechanisms (6–9), while it has also been reported that their activity involves a wide range of non-specific interactions, such as PS ODNs interference of cell growth, cell morphology, viral proliferation, enzyme activity and mRNA expression (10–13).

The enhancement of the intracellular stability and uptake of unmodified ODNs has also been attempted with different systems being used, for example ODN inclusion in liposomes (14–16) and the development of specific carriers or delivery system as tailor-made polymeric nanospheres (17). This latter system gives more reproducible results due to the improved structural and morphological control of the carrier. Indeed, an efficient delivery system has recently been developed (18) based on the use of specifically designed functional nanospheres with controlled size and surface nature. Due to the nanospheres being able to exchange their bromide counterions with the internucleosidic phosphate groups, ODNs can be directly immobilized to the nanosphere surface.

Following our previous works (15,16) on ODNs targeted to the *c-myb* encoded mRNA (codons 2–7), in this paper we describe the immobilization of AS ODN on a core shell nanosphere sample and its biological effects on human leukemia HL60 cells. The cellular uptake of the ODN–nanosphere complexes was determined with FITC and radiolabeled ODNs. Flow cytometry was used to quantify the FITC ODN inside the cells.

Our results show that the core shell nanospheres greatly enhance the cellular uptake of the unmodified AS ODN, which is protected against nuclease degradation in cell culture in medium for up to 8 days.

MATERIALS AND METHODS

Core shell nanosphere preparation

The functional nanosphere sample was prepared by free radical emulsion polymerization of methylmethacrylate (MMA) using quaternary ammonium salt of 2-(dimethylamino) ethyl methacrylate, a reactive emulsifier. In a typical emulsion polymerization reaction, 4 ml of MMA were added to 33 ml of an aqueous solution of the reactive surfactant [2.5% (w/w) with respect to MMA] and free radical initiator potassium persolphate [0.135% (w/w) with respect to MMA]. To eliminate the oxygen from the

*To whom correspondence should be addressed. Tel: +39 06 49852569; Fax: +39 06 49852505; Email: citro@ifo.it

reaction mixture, the microemulsion was frozen by liquid nitrogen and then degassed under 0.665×10^3 Pa (5 Torr) for 3 min. The flask was then charged with nitrogen. This procedure was repeated three times and the flask was then placed in a thermostat bath at 85 ± 1.0°C for 2 h and stirred constantly.

At the end of the reaction the product was filtered and purified by dialysis with an aqueous solution of the appropriate surfactant to remove the remaining monomer. The polymeric nanoparticles were then dried under vacuum at room temperature. The reaction yield was ~80%. The amount of superficial functional groups in the nanospheres was determined by potentiometric titration of the bromine ions obtained after complete ionic exchange of the ammonic groups. The ionic exchange was accomplished by dispersing 0.5 g of the nanospheres in 25 ml of 1 M KNO_3 at room temperature for 48 h in a beaker. The above conditions were found to be optimum for achieving a quantitative ionic exchange of the ammonic groups. The mixture was then adjusted to pH 2 with dilute H_2SO_4 and the bromide ions were titrated with a 0.01 M solution of $AgNO_3$.

Oligonucleotides

The following unmodified phosphodiester oligonucleotides were synthesized: AS ODN, 5′-GTG CC**G GGG** TCT TCG GGC-3′ (free and radiolabeled); FITC ODN, 5′-FITC GTG CC**G GGG** TCT TCG GGC-3′; S ODN, 5′-GCC CGA AGA CCC CGG CAC-3′; SCR1 ODN, 5′-GCT GT**G GGG** CGG CTC CTG-3′; SCR2 ODN, 5′-GTA CC**G GGG** TCC TTG AGC-3′; and INV ODN, 5′-CGG GCT TCT **GGG G**CC GTG-3′.

The oligomers were obtained by automated solid-phase synthesis (Gene Assembler Plus from Pharmacia) by means of β-cyanoethyl-phosphoroamidite chemistry (10 µmol scale) on CPG 500 Å solid supports (ChemGene). The ODNs were purified by anion exchange chromatography (DEAE Sephacel from Pharmacia; triethylammonium-bicarbonate gradient from 0.1 to 1.5 M, pH 7.4). The fractions with HPLC titer >90% were collected, co-evaporated several times with water to remove the excess buffer, converted to the sodium salt (Dowex 50WX8 resin; Na^+ form) and then lyophilized. The UV melting experiments were performed by recording the absorbance at λ = 260 nm of 3 mM solutions of the 1:1 AS ODN–S ODN complex in Tris buffer (0.1 M Tris–HCl, 0.05 M NaCl, pH 7).

Immobilization of ODNs on nanospheres

Nanospheres (10 mg/ml) were dispersed in 20 mM sodium phosphate buffer (pH 7.4) and ultrasonicated for 15 min. The ODN was then added at the appropriate concentration. The suspensions were stirred for 2 h at 25°C. After centrifugation at 13 000 r.p.m. for 3 min, the supernatant was diluted with the appropriate amount of sodium phosphate buffer and the UV absorbance was measured at λ = 260 nm. By subtracting this value from the absorbance value of the added ODNs, we determined the percentage of binding.

For the biological tests, 0.1 µmol of each ODN was added to 1 ml of the sodium phosphate suspension of the nanospheres and continuously stirred for 2 h. After centrifugation at 13 000 r.p.m., the UV absorbance (λ = 260 nm) of the supernatant was measured. The final loading obtained (~10 µmol ODNs/g of nanospheres) corresponds to the quantitative immobilization of the administered ODNs. To remove the excess buffer and to sterilize the complexes, the pellet was washed three times with sterile water, twice with HPLC grade methanol and then lyophilized.

Release experiments of ODNs from the nanospheres

The release of ODNs from the nanospheres (10 µmol ODN/g nanospheres) was monitored at various NaCl concentrations (0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 M) and pH values (5.0, 7.4, 9.0 and 11.0) in the presence of 20 mM sodium phosphate buffer. The ODN release was determined through the comparison of the UV absorbance values (λ = 260 nm) before and after release. In addition, the ODN release from the nanospheres was detected in RPMI 1640 culture medium (Gibco BRL), in the presence or absence of 10% fetal bovine serum (FBS). In a parallel experiment, the release of ODNs from the nanospheres was performed in RPMI 1640 culture medium, in the presence of heat-inactivated (65°C for 30 min) 10% FBS.

HPLC analysis of released ODNs

The composition of ODNs released at different times in culture medium (with or without 10% FBS) was analyzed through anionic exchange HPLC (Toso Haas DEAE 5PW column) at pH 10. Equal amounts of culture medium (in the presence or absence of free or heat inactivated 10% FBS) were first analyzed to allow baseline back-subtraction. According to the amount of released ODNs (previously measured by UV absorption), 25–250 µl of the supernatant were injected. After centrifugation and removal of the supernatant, release of the remaining ODNs immobilized to the nanospheres was achieved through incubation (30 min) in the appropriate volume (10 mg/ml) of 1.0 M NaCl in 20 mM phosphate buffer (pH 7.4). The HPLC analysis was performed with a linear gradient of $NaClO_4$ (from 20 mM to 0.3 M in 30 min) in 20 mM Tris base buffer (pH 10).

Cell cultures

HL60 cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum, 2 mM glutamine and 0.04 mg/ml gentamycin, in a humidified atmosphere with 5% CO_2/95% air at 37°C.

HL60 cell proliferation assays

Cells were seeded in 24-well plates (Nunc) at a concentration of 1×10^4 cells/ml (5×10^3 cells/well), and treated immediately with 16 µM free ODN or with 320 nM of each ODN (either free or immobilized to the nanospheres) dissolved in RPMI 1640 medium. Control cells were grown in the same conditions. Cell counts and viability (trypan blue dye exclusion) were determined daily for up to 8 days of treatment.

ODN uptake by HL60 cells

5′-Dephosphorylated AS ODN was labeled at its 5′ end using T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham) for 30 min at 37°C, according to standard protocols. For the uptake assays, HL60 cells (1×10^4 cells/well) were treated with 5′-end-labeled ODN, free or immobilized to the nanospheres, at a final concentration of 1.4 µM (3×10^6 c.p.m./well). After incubation at 37°C for 2, 9 and 23 h, cells were collected and the ODN uptake was evaluated by using the following two protocols. (i) Cells treated with free radiolabeled AS ODN were centrifuged

at 400 r.p.m. for 5 min to remove the supernatant fraction containing only non-incorporated radiolabeled ODN. The cell pellets were washed twice in ice-cold PBS and the ODN uptake was measured as the radioactivity associated with cells using a Beckman LS6000SC Beta Counter. (ii) Cells treated with radiolabeled AS ODN immobilized on the nanospheres were centrifuged at 400 r.p.m. for 5 min and the supernatant fraction removed. The cells were then lysed by hypotonic shock in bidistilled water and centrifuged at 400 r.p.m. for 5 min to pellet the nanospheres still complexed to the radiolabeled compound. By counting the radioactivity in the lysate fraction (supernatant), we only considered for the uptake evaluation the AS ODN already released into the cells from the nanospheres, and so able to hybridize or already complexed to the target mRNA.

To verify that the experimental conditions employed (hypotonic lysis and further centrifugation) did not cause any release of the immobilized compound from the nanospheres, the same protocol was used on AS ODN immobilized to the nanospheres without the cells as a control.

Flow cytometric analysis

5′-Fluorescein-labeled *c-myb* antisense oligonucleotides (FITC-ODNs) were used for the cytofluorimetric cellular uptake studies. HL60 cells (5×10^5) were incubated with 5 μg/ml FITC-ODNs free or complexed to the nanoparticles at 37 and 4°C for 2 h. This incubation time was chosen since at 4°C cells began dying when exposed for longer periods. After treatment, cells were washed three times with cold PBS containing 0.01% Triton X-100 to remove the FITC-ODNs adsorbed outside the cellular membrane. The cell viability was then determined (trypan blue staining) just before flow cytometric analysis. Twenty-thousand events for each sample were accumulated using a fluorescence-activated cell sorter [FACscan cytofluorimeter (Becton Dickinson, San Jose, CA)], and the list mode data were analyzed using Lysis II-C3.2 Becton Dickinson software.

Western blotting

To evaluate the expression of c-Myb protein in the cultured cell line at days 2 and 4 after treatments, 10^6 cells were incubated at 4°C for 30 min in lysis buffer with ionic detergent (2% SDS, 20 mM Tris pH 8.0, 2 mM PMSF) and then ultrasonicated. Forty micrograms of total proteins were loaded for each sample on a 10% SDS–polyacrylamide gel. Transfer to nitrocellulose (Immobilion PVDF membrane; Millipore Corporation, Bedford, MA) was performed in glycine transfer buffer (192 mM glycine, 25 mM Tris pH 8.8, 20% v/v methanol) for 3 h at 30 V. Western blot was carried out with ECL western blotting kit (Amersham, Arlington Heights, IL). Anti-c-Myb mAb (UBI 05-175) was used at 1:1000 dilution. Peroxidase-labeled anti-mouse antibody NF-825 (Amersham) was used according to manufacturer's instructions. To check the protein amount transferred to nitrocellulose membrane, heat-shock protein (HSP) was used as control and detected by an anti-human HSP 72/73 mAb (Ab-1, clone W27; Calbiochem, Cambridge, MA). The relative amounts of the transferred c-Myb protein were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad) and normalized to the related HSP 72/73 amounts.

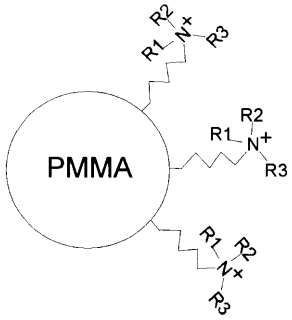

Figure 1. Schematic chemical structure of PMMA nanospheres.

RESULTS

Core shell nanosphere preparation and characterization

The functional nanospheres were prepared by free radical emulsion polymerization of MMA using a reactive emulsifier consisting of a methacrylic monomer and a quaternary ammonium group with a long alkyl substituent. Due to the preferential localization of the emulsifier at the interface between the monomer and water, it is likely that a function outer shell of quaternary ammonium groups anchored to an inner polymethylmethacrylate core shell structure was formed (Fig. 1). The nanosphere sample employed in the present study has an average diameter of 480 ± 50 nm (Fig. 2) and a surface density of ammonium groups of 18 μmol/m^2.

Synthesis and purification of the ODNs

The following 18mer unmodified oligodeoxynucleotides were used: AS ODN, 5′-GTG CC**G GGG** TCT TCG GGC-3′ (together with its 5′-FITC and radiolabeled derivatives) directed against the codons 2–7 of *c-myb* encoded mRNA, which inhibit human leukemia HL60 cell proliferation (16).

The following four ODNs were used as control sequences: S ODN, 5′-GCC CGA AGA CCC CGG CAC-3′; SCR1 ODN, 5′-GCT GT**G GGG** CGG CTC CTG-3′; SCR2 ODN, 5′-GTA CC**G GGG** TCC TTG AGC-3′; INV ODN, 5′-CGG GCT TCT **GGG G**CC GTG-3′.

All ODNs were purified by anion exchange chromatography and converted to their sodium salts. UV melting analysis of the 1:1 complex AS:S revealed a very cooperative melting process (T_m = 75°C).

Immobilization of ODNs on the nanospheres

All ODNs were able to bind to the core shell nanospheres through the interaction between the internucleosidic phosphate groups and the positively charged ammonium groups at the surface of the nanospheres. Incubation of increasing amounts of ODNs with 10 mg/ml of nanospheres in 20 mM sodium phosphate buffer (pH 7.4) allowed increased values of final loading, ranging from 0.2 to 12 μmol ODN/g of the nanospheres (Fig. 3).

The binding evidently occurred through an ionic interaction mechanism since the adsorption of the ODNs on a comparable nanosphere sample without any ammonium group on the surface was negligible. Moreover, incubation in buffers with salt

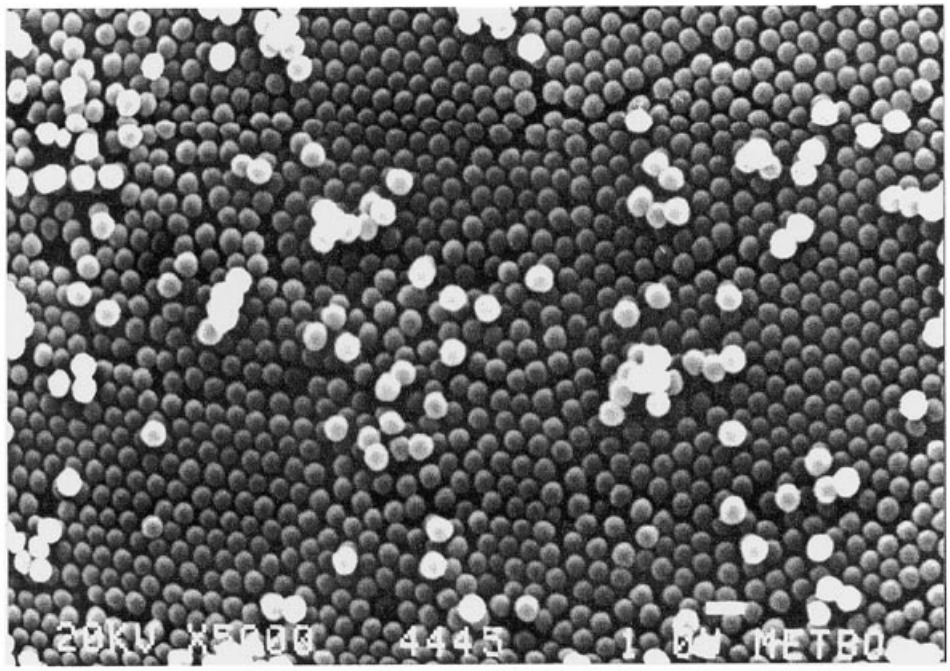

Figure 2. Scanning electron micrograph of the nanosphere sample. The order of magnification is indicated by the reported bar.

concentrations >20 mM reduced the capacity of the ODNs to bind onto the nanospheres.

Release of the ODNs from the nanospheres

The ODN release from the nanospheres closely depends on the ionic strength of the medium and was nearly complete in the presence of 1.0 M NaCl. This behavior was pH insensitive in the 5–10 pH range. Accordingly, as expected, the release in RPMI 1640 culture medium at 37°C after 24 h was minimum (4.5%), due to the low ionic strength, but increased in the presence of 10% FBS and 10% heat-inactivated FBS to reach the values of 14 and 24%, respectively, after 24 h.

HPLC analysis of released AS ODN in RPMI 1640 culture medium

HPLC analysis was performed on the AS ODN released after days 1, 2 and 5. As expected, after 1 day's incubation in RPMI 1640 culture medium, extensive degradation of the released unmodified ODN occurred in the presence of 10% FBS serum, whereas release of mainly intact ODN could be detected in the presence of 10% heat-inactivated FBS serum. After centrifugation and removal of the supernatant, the AS ODN still immobilized to the nanospheres was recovered in the presence of 1.0 M NaCl buffer. The anionic exchange HPLC analysis revealed that the AS ODN, previously immobilized on the nanospheres, was still intact after 5 days of incubation in the presence of native FBS (Fig. 4).

Cellular uptake of free AS ODN and AS ODN reversibly immobilized on the nanospheres

The trend of cellular uptake, as a function of time, of free AS ODN and AS ODN reversibly immobilized on the nanospheres is illustrated in Figure 5. The uptake of the free ODN was ~0.01% after 2 h, and remained between 0.05 and 0.15% for up to 23 h.

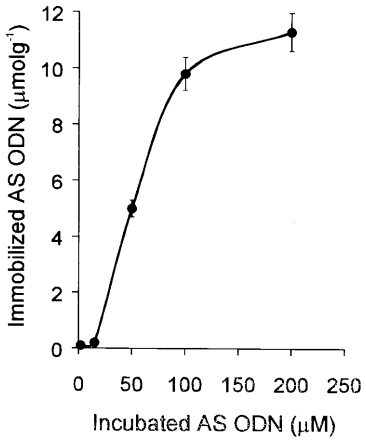

Figure 3. Trend of the AS ODN immobilization per gram of nanospheres as a function of the concentration of the incubated ODN. Each point represents the mean of three observations (±SD).

In the case of the ODNs reversibly immobilized on the nanospheres, the cellular uptake increased sharply at first and then more gradually to reach a plateau value of 10% after 10 h.

Flow cytometry

ODN–nanosphere uptake was evaluated by incubating HL60 cells with FITC-ODN either free or complexed to nanoparticles at 4 and 37°C, and the fluorescence intensity associated with treated cells was measured by flow cytometry. To distinguish the energy-dependent FITC-ODN uptake from the FITC-ODN passive diffusion through the cellular membrane, the experiments were carried out at 4 and 37°C. The fluorescence intensity associated with cells exposed to 5 μg/ml of free or NP-associated FITC-ODN for 2 h is shown (Fig. 6). No significant change in

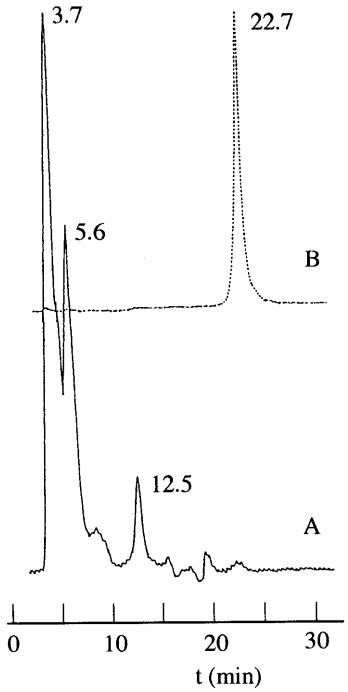

Figure 4. Anion exchange HPLC traces of (A) oligonucleotidic fragments present in RPMI 1640 culture medium containing 10% FBS after 24 h of incubation at 37°C of the AS ODNs immobilized on the nanospheres, and of (B) AS ODN chemically recovered from the nanospheres after 5 days of incubation at 37°C in RPMI 1640 culture medium containing 10% FBS.

fluorescence intensity was observed in the cells treated with free FITC-ODN when endocytosis was prevented by incubation at 4°C, compared with untreated control cells (Fig. 6A). The difference of the fluorescence intensity associated with the cells treated with free FITC-ODN both at 37° (Fig. 6B) and 4°C (Fig. 6A) was small after only 2 h of incubation. On the contrary, the treatment of HL60 cells with FITC-ODN complexed to the nanoparticles at 37°C (Fig. 6D) caused a significant increase of the fluorescence intensity associated with the cells, as compared with that observed either when the same treatment was carried out at 4°C (Fig. 6C) or when the cells were exposed to the free FITC-ODN at 37°C (Fig. 6B). These data suggest that an energy-dependent mechanism is likely to be involved in the uptake of NP-associated FITC-ODN. In addition, the entry of the ODN–nanosphere complexes into cells is more rapid then the entry of free ODNs under the same experimental conditions. After the flow cytometric analysis, the cells were cyto-centrifuged and the fluorescence localization was evaluated by fluorescence microscopy. The spots of fluorescence were only detectable in the cytoplasm of cells treated at 37°C with NP-associated FITC-ODN; only a small amount was found to be close to the plasma membrane (data not shown). The cell viability after 2 h of incubation with free or NP-associated FITC-ODN was >85%, indicating the absence of toxic effects during all treatments employed.

Effect on HL60 cell proliferation of AS and control ODNs immobilized on the nanospheres

AS ODN targeted to the codons 2–7 of *c-myb* mRNA inhibited HL60 cell proliferation when used at a concentration of 16 μM (Fig. 7A), confirming our previous data (15). To evaluate whether

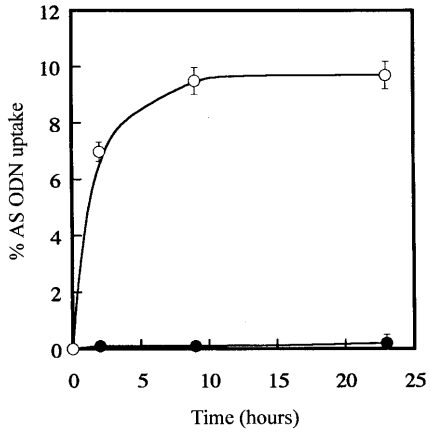

Figure 5. Cellular uptake of free and immobilized ODN by HL60 cells as a function of time. HL60 cells were incubated at 37°C with 5′-labeled ODN free (filled circles) or immobilized 5′-labeled ODN (open circles). At the indicated time, cellular uptake was monitored as the ratio of radioactivity in cellular lysate versus total radioactivity. Each point represents the mean of three observations (±SD).

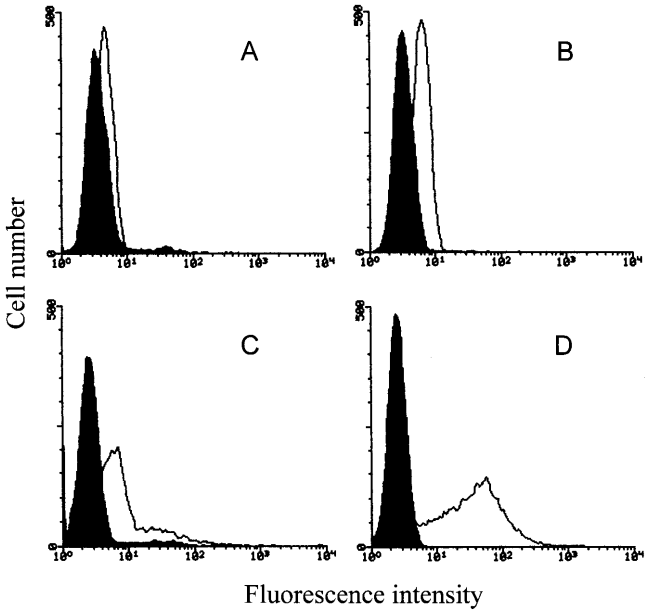

Figure 6. Flow cytometric quantification of FITC-ODN internalization. HL60 cells were treated with free FITC-ODN (5 μg/ml) at 4° (**A**) and 37°C (**B**) for 2 h. The same treatments were performed with FITC-ODN (5 μg/ml) adsorbed to nanospheres at 4° (**C**) and 37°C (**D**). The black area represents the auto-fluorescence intensity of untreated cells.

the delivery of ODNs allows the use of low concentrations of AS ODNs while at the same time inducing the same inhibition of cell proliferation observed with 16 μM free AS ODNs, we performed dose–response experiments starting with at least 1 log less ODNs (i.e. 1.6 μM) adsorbed on NP (Fig. 7B). When the cells were treated with AS ODN immobilized to the nanosphere at concentration of 320 nM, the inhibitory effect on HL60 cell proliferation was outstanding and comparable with that observed when a 50-fold higher concentration of free AS ODN (16 μM) was employed (Fig. 7C). Treatments with free AS ODN as well as S ODN, SCR1 ODN, SCR2 ODN and INV ODN at a concentration of 320 nM, were not able to induce any change on the cell proliferation rate. Since the results obtained with all the

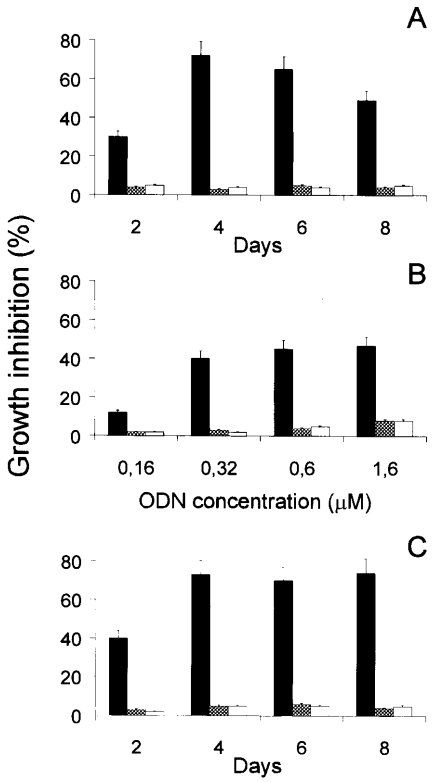

Figure 7. Effect of free and immobilized *c-myb* ODN on HL60 cell proliferation. Values are expressed as percent of inhibition of cell proliferation related to untreated cell. (**A**) HL60 cells treated with free AS ODN. Black bars, 16 μM AS ODN; gray bars, 320 nM AS ODN; white bars, 320 nM free SCR1 ODN. (**B**) HL60 cells treated with 0.16, 0.32, 0.6 or 1.6 μM immobilized AS ODN (black bars), immobilized SCR1 ODN (gray bars) or correspondent amount of free nanospheres (white bars). (**C**) HL60 cells treated with 320 nM immobilized AS ODN (black bars), 320 nM immobilized SCR1 ODN (gray bars) and free nanospheres (white bars) at the same concentration (30 ng/ml) used for the treatments with immobilized ODNs. Results obtained with the other control ODN treatments were similar to that of SCR1 ODNs. Each point represents the mean of triplicate samples (±SD).

control sequences used were found superimposable, only the data referring to SCR1 ODN were reported (Fig. 7A). The treatment with AS ODN immobilized to the nanospheres resulted in a longer inhibition of cell proliferation, even though the AS ODN administration was performed in a single dose at the beginning of the experiment. In fact, the inhibition was ~40% at day 2 (in comparison with untreated cells), and remained at 70–75% up to day 8, whereas a decrease of the inhibitory effect was observed after day 4 with treatment with the free 16 μM AS ODN. The sustained effect of the AS ODN immobilized to the nanospheres suggests that the nanospheres protected the ODNs from nuclease degradation. The addition of 320 nM NP-associated S ODN and G-quartet containing SCR1, SCR2 and INV ODNs, to HL60 cells did not result in any inhibitory effect on the cell proliferation rate and the same results were obtained with the exposure of the cells to the free NP; only the data referring to SCR1 ODN treatment are reported (Fig. 7C).

Expression of c-Myb protein in HL60 cells treated with AS and SCR1 ODNs immobilized on the nanospheres

The western blots of c-Myb protein expression in HL60 cells exposed to AS and SCR1 ODNs immobilized on the nanospheres and to free nanospheres after days 2 and 4 are reported (Fig. 8). On day 2, cells treated with immobilized AS ODN showed a reduction in c-Myb protein level of ~24% (in comparison with the control cells), as evaluated by densitometric analysis and normalization to HSP 72/73 level. A still greater reduction in c-Myb protein level was detected on day 4, with a 59% decrease in protein expression induced by AS ODN immobilized to the nanospheres. No significant change in c-Myb protein level was observed, on either day 2 or 4, in cells exposed to the control sequence SCR1 immobilized to the nanospheres and to the free nanospheres. These results indicate that the inhibitory effect on HL60 cell proliferation was due to the down-modulation of the c-Myb protein level, determined by the sequence-specific reaction of the AS ODN with *c-myb* mRNA.

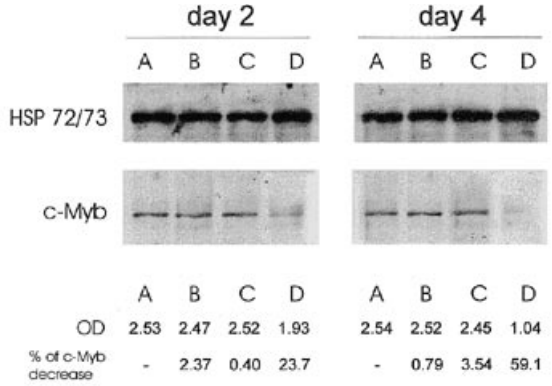

Figure 8. Western blot analysis of c-Myb protein expression in HL60 cells exposed to immobilized ODNs. Analysis performed at days 2 and 4 after a single dose treatment. A, control cells; B, cells treated with free nanospheres; C, cells treated with immobilized SCR1 ODN; D, cells treated with immobilized AS ODN. HSP 72/73 expression served as control for uniformity of protein gel loading and blotting. In the bottom of the figure the densitometric quantifications of the c-Myb protein normalized by HSP are reported.

DISCUSSION

A variety of systems have been developed to overcome the rapid degradation of ODN in biological media, which is the major drawback to a greater commercial use of the antisense approach.

Lipophylic polyalkylcyanoacrilate nanospheres, developed for the delivery of doxorubicin, are able to reversibly bind antisense oligonucleotides, in the presence of high concentrations of a hydrophobic quaternary ammonium salt (cetyltrimetyl-ammonium bromide, CTAB), which allow ion-pair formation (19). Unfortunately, the release of ODN is accompanied with the concomitant release of CTAB, which, at high concentrations, induces toxicity in cells. Cholesterol-modified ODNs, capable of directly interacting with nanospheres without the need for potentially toxic intermediates, have also been employed, but a lower inhibition of human tumor cells proliferation was found compared with the previously described system (20). Diethyl-aminoethyl-dextran stabilized PHCA nanoparticles improved the cellular uptake of antisense oligonucleotides (21) but, after incubation in the cellular medium, a protective coating of the complex by serum proteins prevented oligonucleotide release from the nanospheres.

To obtain an efficient delivery of ODNs through biological membranes and so a reduction in the ODN dosage, we prepared

novel polymeric nanospheres, specifically designed to directly bind polyanionic antisense agents without requiring other potentially toxic intermediates. We were able to modulate the amount of unmodified phosphodiester ODNs (0.2–12 μmol ODNs/g) immobilized on their surface. Release was easily obtained in the presence of high NaCl concentration, whereas it was minimum in the presence of the culture medium, thus preventing loss of active compound outside cells. Binding to the nanospheres seems to prevent ODNs from nuclease degradation so increasing their half-life inside cells.

Moreover, AS ODN delivery resulted in a considerable reduction (50-fold) in the amount of oligomers (320 nM) required to inhibit cell proliferation to the same extent of that observed with 16 μM free AS ODNs. Furthermore, this amount of ODNs was delivered by 33 ng/ml of nanospheres, 5-fold less than the maximal dose non-toxic used. Interestingly, the order of magnitude of the increase in uptake between free and NP-associated AS ODNs is similar to the reduction of AS ODN dosage obtained with our nanospheres. This suggests a close correlation between the antitumor effect and the cellular uptake. A substantially longer bioavailability of the intact ODNs inside cells is also probably involved.

The reduction of cellular proliferation is mainly due to an antisense mechanism as no effect is detectable when the control sequences are immobilized on the same sample of nanoparticles. In particular, our nanospheres are also able to minimize the side effects of G-quartet ODNs, which can occur due to the aspecific interaction of guanosine quadruplex structures with other cellular targets. These interactions mainly seem to occur with high concentrations of fully phosphorothioate modified sequences that contain at least four guanines in a row (13), and are particularly important when the ODNs are targeted to mRNAs coding for proteins involved in cell growth and proliferation. On the other hand, these quadruplex structures show different stability depending on the cellular systems and physiologically relevant salt and temperature conditions (22).

The lack of G-quartet effects in our ODNs, when immobilized on the described PMMA particles, could be due to: (i) the absence of phosphorothioate chemical modifications (13); (ii) the reduced ability of ODNs to form homotetrameric quadruplex structures due to the low concentrations of active immobilized ODNs. In fact, most of the reported aspecific antiproliferative activity of G-rich ODNs was only observed at high ODN concentrations as it involves an intermolecular association (23); or (iii) the interaction of ODNs with the nanoparticle's surface, which could prevent interstrand aggregation.

A single dose of immobilized *c-myb* antisense ODN allows an efficient sequence-specific inhibition of the cell proliferation even after 8 days. Since only the AS ODNs are able to inhibit cell growth, we can hypothesize that the release of antisense ODNs from nanospheres is due to the binding affinity of ODNs with *c-myb* mRNA. Given that the level of c-Myb protein was decreased by ~24% on day 2 and ~60% on day 4, it also appears that the activity of the AS ODNs is the result of a continuous release of intact ODNs from the nanospheres to the cells, which simulates a continuous infusion administration.

Further experiments are in progress in order to verify the effectiveness of our new polymeric carrier for antisense ODNs in other cellular and animal systems. In fact, due to the synthetic method, the nanosphere surface nature can be modulated in order to improve the bioavailability of antisense agents and other anticancer and antiviral agents, thus opening a new and reliable general strategy for increasing the cellular uptake and the biological stability of ODNs.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Anna Garbesi for continuous and helpful discussions. We also acknowledge the help of Dr Federica Sentinelli for experimental design and assistance, and Mrs Simona Righi for assistance in preparing the manuscript. This work was partially supported by grants from Associazione Italiana per la Ricerca sul Cancro, Consiglio Nazionale delle Ricerche (to G.C.). A.R. was supported by a fellowship from FIRC, Fondazione Italiana per la Ricerca sul Cancro.

REFERENCES

1 Milligan,J.F., Matteucci,M.D. and Martin,J.C. (1993) *J. Med. Chem.*, **36**, 1923–1937.
2 Zon,G. (1995) *Mol. Neurobiol.*, **10**, 219–229.
3 Leonetti,C., D'agnano,I., Lozupone,F., Valentini,A., Geiser,T., Zon,G., Calabretta,B., Citro,G., Zupi,G. (1996) *J. Natl Cancer Inst.*, **88**, 419–429.
4 Citro,G., D'agnano,I., Leonetti,C., Perini,R., Bucci,B., Zon,G., Calabretta,B. and Zupi,G. (1998) *Cancer Res*, **58**, 283–289.
5 Beltinger,C., Saragovi,H.U., Smith,R.M., Lesauteur,L., Shah,N., Dedionisio,L., Christensen,L., Raible,A., Jarett,L. and Gewirtz,A.M. (1995) *J. Clin. Invest.*, **95**, 1814–1823.
6 Chiang,M.Y., Chan,H., Zounes,M.A., Freier,S.M., Lima,W.F. and Bennett,C.F. (1991) *J. Biol. Chem.*, **266**, 18162–18171.
7 Lisziewicz,J., Sun,D., Klotman,M., Agrawal,S., Zamecnik,P. and Gallo,R. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11209–11213.
8 Ratajczak,M.Z., Kant,J.A., Luger,S.M., Hijiya,N., Zhang,J., Zon,G. and Gewirtz,A.M. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11823–11827.
9 Higgins,K.A., Perez,J.R., Coleman,T.A., Dorshkind,K., Mccomas,W.A., Sarmiento,U.M., Rosen,C.A. and Narayanan,R. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 9901–9905.
10 Majumdar,C., Stein,C.A., Cohen,J.S., Broder,S. and Wilson,S.H. (1989) *Biochemistry*, **28**, 1340–1346.
11 Ghosh,M.K., Ghosh,K. and Cohen,J.S. (1992) *Antisense Res. Dev.*, **2**, 111–118.
12 Gao,W.Y., Han,F.S., Storm,C., Egan,W. and Cheng,Y.C. (1992) *Mol. Pharmacol.*, **41**, 223–229.
13 Yaswen,P., Stampfer,M.R., Ghosh,K. and Cohen,J.S. (1993) *Antisense Res. Dev.*, **3**, 67–77.
14 Bertling,W.M., Gareis,M., Paspaleeva,V., Zimmer,A., Kreuter,J., Nurberg,E. and Harrer,P. (1991) *Biotech. Appl. Biochem.*, **13**, 390–405.
15 Citro,G., Perrotti,D., Cucco,C., D'agnano,I., Sacchi,A., Zupi,G. and Calabretta,B. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 7031–7035.
16 Citro,G., Szczylik,C., Ginobbi,G., Zupi,G. and Calabretta,B. (1994) *Br. J. Cancer*, **69**, 463–467.
17 Chavany,C., Le Doan,T., Couvreur,P., Puisieux,F. and Hélène,C. (1992) *Pharm. Res.*, **9**, 441–449.
18 Laus,M. and Tondelli,L. (1997) Italian Patent no. BO97A000641.
19 Schwab,G., Chavany,C., Duroux,I., Goubin,G., Lebeau,J., Hélène,C. and Saison-Behmoaras,T. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 10460–10464.
20 Godard,G., Boutorine,A.S., Saison-Behmoaras,E. and Hélène,C. (1995) *Eur. J. Biochem.*, **232**, 404–410.
21 Zobel,H.P., Kreuter,J., Werner,D., Noe,C.R., Kumel,G. and Zimmer,A. (1997) *Antisense Nucleic Acid Drug Dev.*, **7**, 483–493.
22 Basu,S. and Wickstrom,E. (1997) *Nucleic Acids Res.*, **25**, 1327–1332.
23 Burgess,T.L., Fisher,E.F., Ross,S.L., Bready,J.V., Qian,Y.-X., Bayewitch,L.A., Cohen,A.M., Herrera,C.J., Hu,S.S., Kramer,T.B., Lott,F.D., Martin,F.H., Pierce,G.F., Simonet,L. and Farrell,C.L. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 4051–4055.