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Research paper

Uptake of oligonucleotide-loaded nanoparticles in prostatic cancer cells and their intracellular localization

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Abstract

The development of antisense biotechnology is dependent, in part, on creating improved methods for delivering oligonucleotides to cells. In this study, we investigated a colloidal system (nanoparticles (NP) of poly (D,L) lactic acid) that affects the intracellular delivery of oligonucleotides. We have examined the intracellular compartmentalization in DU145 cells of fluorescein labeled phosphorothioate oligonucleotides, both in the free state and when loaded into NP. Fluorescent oligonucleotides were incubated for 18 h with DU145 cells and the mean intracellular fluorescence was determined by flow cytometry. After the addition of monensin, an increase in signal intensity was observed, indicating that free oligonucleotides were resident in an acidic intracellular environment, whereas oligonucleotides from the NP did not reside in an acidic compartment. Free and NP loaded with oligonucleotides effluxed from DU145 cells from two intracellular compartments. This preliminary report indicates that colloidal carriers such as NP could prove to be useful in affecting intracellular trafficking of oligonucleotides in DU145 and in other cells.

Keywords: Oligonucleotides; Nanoparticles; Poly (D,L) lactic acid; Cellular uptake; Intracellular compartmentalization

1. Introduction

Prostate cancer kills 41 000 patients in the US per year and is the leading cancer diagnosed in men in the Western world [1]. Treatment is primarily hormonal, but when patients fail to respond, the average life expectancy is measured in months [2]. Obviously, novel treatments are needed, which the antisense approach may provide. This approach is based on the use of synthetic antisense oligonucleotides that can selectively inhibit gene expression and thus block the production of the corresponding protein. However, although antisense oligonucleotide efficacy has been demonstrated in vitro, suboptimum stability and insufficient cellular uptake, as well as a lack of understanding of their intracellular pharmacology, limit their development as anticancer or antiviral therapies [3].

Different kinds of vectors have been proposed as means to solve these drawbacks [4–8]. For instance, the incorporation of oligonucleotides into colloidal polymeric carriers, such as nanoparticles (NP), should not only improve their stability and their uptake, but might also modify the intracellular localization of the oligonucleotides. The objectives of the present work were to formulate a model system using a non-sequence-specific oligonucleotide to evaluate the nanoparticle concept. We then prepared oligonucleotide-loaded biocompatible NP and evaluated their uptake and efflux from DU145 prostatic cancer cells.

2. Materials and methods

2.1. Synthesis and labelling of oligonucleotides

A 15-mer phosphorothioate homopolymer of thymidine (SdT15) was labeled at the 5′-end using fluorescein isothio-

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cyanate (FITC, Molecular Probes, Eugene, OR) (F-oligonucleotides) as previously described [9].

2.2. Production of an oligonucleotide hydrophobic complex

A hydrophobic complex of F-oligonucleotides was prepared by complexation with a cationic detergent, cetyltrimethylammonium bromide (CTAB, Fluka chemica, Buchs, Switzerland). Practically, a 7.5% (m/v) CTAB was prepared in distilled water. 1.5 μl of CTAB solution was added to 1624 μg of F-oligonucleotide in a volume of 58 μl of distilled water and mixed gently. The mixture was centrifuged for 3 min at 12 000 rpm. Then, without disruption of the pellet residue, 0.5 μl of CTAB solution was added to the supernatant and the mixture was again centrifuged for 3 min at a speed of 12 000 rpm. Further 0.5 μl aliquots of CTAB solution were added and the sample centrifuged until no cloudiness could be seen in the supernatant. The sample was then centrifuged for 15 min at 12 000 rpm and the supernatant discarded. Then, the pellet residue containing the F-oligonucleotide-CTAB complex was dried under vacuum.

2.3. Preparation of oligonucleotides-loaded NP

NP were prepared by the emulsification-diffusion method [10]. The total amount of dried F-oligonucleotide-CTAB complex (3.25% initial oligonucleotide loading) was dissolved in 40 μl benzyl alcohol and added to 1.06 ml of a solution containing 5% poly(D,L-lactic acid), (PLA, Medisorb 100DL) in benzyl alcohol. 2 ml of an aqueous solution of 15% poly(vinyl alcohol) were then added and the mixture was stirred at 1400 rpm using a mechanical stirrer fitted with a four paddle attachment. Subsequent dilution of the emulsion with 33 ml of distilled water induced the formation of the NP. The NP suspension was then purified by three centrifugation steps at a speed of 15 000 g for 10 min and finally freeze-dried for 2 days using a LSL Secfroid lyolab BII (LSL Secfroid, Aclens, Switzerland). The mean size of the NP was 375 nm and was determined using a Nanosizer® (Coulter Electronics, Harpenden, Herfordshire, UK).

2.4. Cell culture

DU145 cells were obtained from American Type Culture Collection (Rockville, MD 20852). Cells were grown and maintained in a 5% CO_2 humid environment, in RPMI 1640 medium (Gibco-BRL, Grand Island, NY), containing 10% (v/v) foetal calf serum (FCS), supplemented with 0.1 mM MEM non-essential amino acids, 1 mM pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. The serum was heat inactivated at 65°C for 1 h.

2.5. Internalization experiments

DU145 cells were plated at a density of 5×10^4 cells/well in 24-well plates and incubated for 18 h. The effect of NP concentration and incubation time on F-oligonucleotide internalization was studied. Because a 28-mer phosphorothioate homopolymer of cytidine (SdC28) competes for polyanion binding sites on the cell surface, it was used at a concentration of 10 μM for 5 min to strip off cell surface bound F-oligonucleotides. The cells were then washed twice with cold PBS/BSA, and cold trypsin was added for 30 min. Subsequently, the cells were suspended in trypsin and treated for flow cytometry experiments, as described below.

2.6. Efflux experiments

After a 10 h internalization period, in the presence of free or F-oligonucleotide-loaded NP, as described above, the DU145 cells were placed on ice at 4°C, and the medium was removed. Ten micromolar SdC28 was added for 5 min. Then the medium was removed, replaced by new prewarmed medium and placed in the incubator for various efflux times (1–24 h). At the end of this period of time, the cells were then washed twice with cold PBS/BSA, and resuspended in cold trypsin. They were then treated for flow cytometry experiments, as described below.

2.7. Curve fitting

The efflux data obtained in the manner described above were fitted to an exponential function by GraFit software (Erithacus Software, UK). Each curve was assumed to represent either a mono or biexponential function of the form of $C_T = Ae^{-\alpha t}$ or $C_T = Ae^{-\alpha t} + Be^{-\beta t}$, which corresponds to a monocompartmental or bicompartmental cellular model, respectively. A and B represent the proportion of F-oligonucleotides in each compartment at $t = 0$, and α and β are the rate constants for the loss of F-oligonucleotides from each compartment. All four parameters were determined by multiple iterations so as to minimize the residuals, the only restriction being $A, B, \alpha, \beta > 0$.

2.8. Flow cytometry

Following the internalization or efflux periods, the cells were washed twice with cold PBS/BSA, resuspended in 200 μl PBS/BSA and added to 100 μl PBS/BSA containing 0.3 μg/ml propidium iodide. The mean fluorescence intensity for a population of 5000 cells was determined on a Becton–Dickinson FACscalibur dual-laser flow cytometer using Cell Quest software, as previously described [9]. Mean fluorescence channel intensities were expressed as the average of these measurements ± SD ($n = 3$). Following the analysis, an adequate amount of monensin (Sigma, St Louis, MO) was added to each tube in order to reach a final concentration of 20 μM. The mean fluorescence intensity for a population of 5000 cells was determined within a time frame of 15–30 min.

3. Results and discussion

3.1. Internalization of F-oligonucleotide-loaded NP

In order to determine the amount of internalized F-oligonucleotide-loaded NP, any free surface bound F-oligonucleotides were removed by competition with an unlabeled oligonucleotide after the incubation period. Then, the cells were analyzed by flow cytometry in the absence or presence of monensin. Flow cytometry analysis demonstrated that F-oligonucleotide-loaded NP entered the DU145 cells in a dose dependent-manner (Fig. 1).

Entry into the cells remained unsaturated at least up to a concentration of 1 mg/ml NP, as shown in Fig. 1. In an attempt to determine the intracellular localization of the F-oligonucleotide-loaded NP, monensin [11], a sodium ionophore that disrupts the pH gradient between the endosome and the cytoplasm, was used. Since the emission intensity of fluorescein is pH dependent, it is possible to determine qualitatively if an internalized F-molecule resides in an acidic intracellular environment. Thus, if the oligonucleotide was resident in a compartment of low pH, the addition of monensin should cause a measurable increase in signal [12]. Only a small increase was observed in signal intensity when cells incubated with F-oligonucleotide-loaded NP were treated with monensin. These data suggest that either these carriers do not reside in an acidic compartment or that acidification is blocked in the presence of NP. The issue was addressed by an experiment performed with blank NP incubated, in DU145 under identical conditions, in the presence of free F-oligonucleotides. In this case, the efflux profile was similar to that obtained with free F-oligonucleotides both in the presence, and in the absence, of monensin (data not shown). This confirms the hypothesis that unloaded NP do not block the increase in endosomal pH due to monensin.

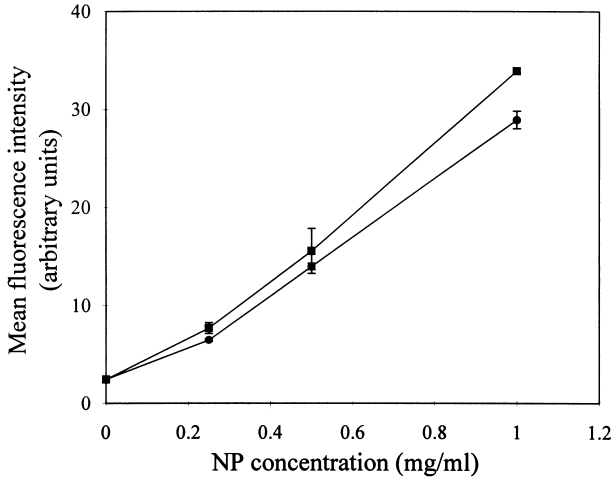

Fig. 1. Internalization of various amount of NP/cell in DU145 cells for 24 h, at 37°C, in the absence (●) or presence (■) of monensin. (mean ± SD, $n = 3$) with different concentrations of NP: 0 μg/ml, 250 μg/ml (containing 0.48 μM of F-oligonucleotide), 500 μg/ml (containing 0.96 μM of F-oligonucleotide) and 1 mg/ml (containing 1.92 μM of F-oligonucleotide).

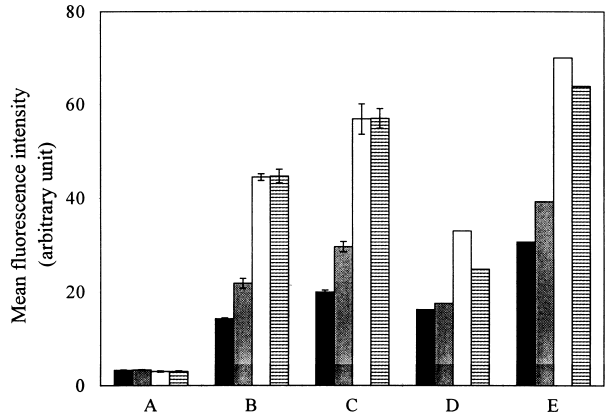

Fig. 2. Internalization of oligonucleotide-loaded-NP or free oligonucleotide in DU145 cells as a function of time (black bars = 6 h, grey bars = 10 h, white bars = 24 h, shaded bars = 30 h), at 37°C, in the absence or presence of monensin, with A = untreated cells (mean ± SD, $n = 3$), B = 750 μg/ml of NP containing 1.44 μM of F-oligonucleotide (mean ± SD, $n = 3$), C = 750 μg/ml of NP containing 1.44 μM of F-oligonucleotide treated with 20 μM of monensin (mean ± SD, $n = 3$), D = 1.44 μM of free F-oligonucleotide (mean, $n = 2$) and E = 1.44 μM of free F-oligonucleotide treated with 20 μM of monensin oligonucleotide (mean, $n = 2$).

The internalization process was then studied as a function of time (6–30 h) with a non-saturating concentration of NP (750 μg/ml, Fig. 2). The intensity of the signal due to internalized F-oligonucleotide-loaded NP increased as a function of time up to 24 h. The overall uptake of free F-oligonucleotides was lower than for F-oligonucleotide-loaded NP. In contrast to the F-oligonucleotide-loaded NP, the fluorescence signal of free F-oligonucleotides was significantly increased in the presence of monensin due to their intracellular localization in an acidic environment, corresponding to endosomes/lysosomes. These results indicate that either the free and F-oligonucleotide-loaded NP internalization pathways, or their intracellular compartmentalization, is likely to be different. It is thus possible that the F-oligonucleotide originating from NP, which did not reside in an acidic compartment, might be located in the cytoplasm/nucleus rather than in endosomes, unlike the non-encapsulated molecules.

3.2. Efflux of F-oligonucleotide-loaded NP from DU145 cells

Efflux experiments were performed to determine the rate at which F-oligonucleotides underwent exocytosis from DU145 cells. These studies were performed after a 10 h internalization period. A representative plot of rate of loss of fluorescence from DU145 cells was determined as a function of time (Figs. 3 and 4). Tonkinson et al. [11] applied a bi-exponential model to the efflux rate. In the present work, the loss of F-oligonucleotides was also fitted to a bi-exponential function of the form $C_T = Ae^{-\alpha t} + Be^{-\beta t}$. As already described [11], the individual exponential terms may be referred to as compartments but do not necessarily correspond to biological structures, although based on confocal

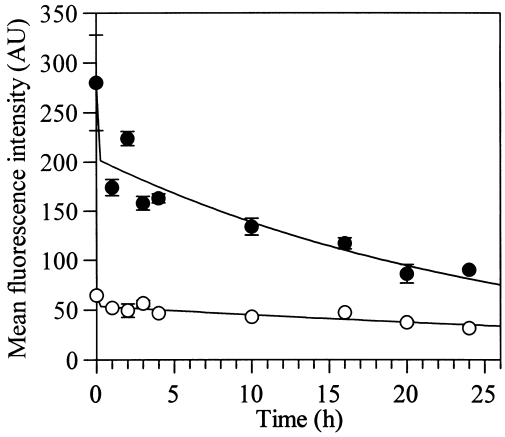

Fig. 3. Efflux of free F-oligonucleotide in the absence (white symbol) or presence (black symbol) of monensin, from DU145 cells after 10 h of internalization. (mean ± SD, $n = 3$).

microscopy studies, they are either endosomes or lysosomes. The coefficients A and B represent the proportion of F-oligonucleotide in each compartment (total = 100%) at the beginning of the efflux experiment. The terms α and β represent the kinetic constants for the rate of loss of material from the compartments. The results are presented in Table 1.

According to the previously described model [11], in the absence of monensin, 84% of the free phosphorothioate F-oligonucleotide was found in the deep (slow efflux) compartment and 16% in the shallow (rapid efflux) compartment. However, since the first phase is based only on two experimental points which is not sufficient (Fig. 3), a mono-exponential curve fitting function was then employed. The values obtained (Table 1) were similar to those obtained previously for B and β with the bi-exponential model. Thus, the efflux of free F-oligonucleotides from the cells was quite slow ($\alpha = 0.02$, $t_{1/2} = 33$ h). As shown in the Table 2, which gives the area under the normalized fluorescence-time curve (AUC) values of the efflux profiles, the

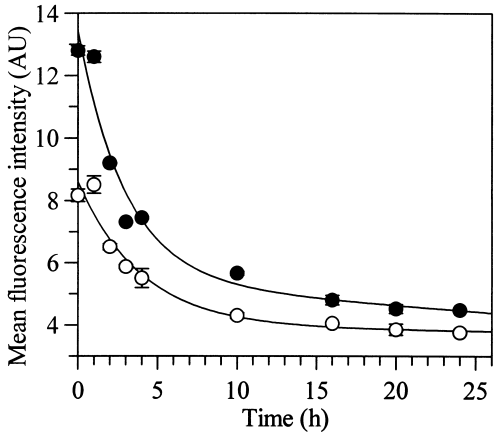

Fig. 4. Efflux of F-oligo in the absence (white symbol) or presence (black symbol) of monensin, from DU145 cells after 10 h of incubation with F-oligonucleotide-loaded NP, (mean ± SD, $n = 3$).

Table 1

Normalized[a] parameters for the bi-exponential loss of F-oligonucleotide loaded in NP and bi-exponential or mono-exponential loss of free F-oligonucleotide from DU145 cells

	A	α	B	β
F-oligonucleotide-loaded NP (bi-exponential)	57	0.25	48	0.001
Free F-oligonucleotide (bi-exponential)	16	29	84	0.01
Free F-oligonucleotide (mono-exponential)	87.59	0.02	–	–

The coefficients A and B represent the percentage of F-oligonucleotide in each compartment following a 10 h incubation; α and β are the rate constant (h^{-1}) for the loss of F-oligonucleotide from each compartment.
[a]To allow comparison of profiles, the experimental data have been normalized. A value of 100 was attributed to the initial $t = 0$ time point.

addition of monensin induced a sharp increase in the fluorescent signal of internalized free F-oligonucleotides. This behaviour is typical of a substance located either initially in an acidic compartment or, as stated by Tonkinson et al. [11] in a deep compartment. This was also in agreement with the $t_{1/2}$ value.

With respect to F-oligonucleotide-loaded NP, Fig. 4 shows that efflux of the F-oligonucleotide followed a bi-exponential decay. According to Tonkinson et al., the A and B values (Table 1) represent, respectively, the proportion of F-oligonucleotide located at the beginning of the efflux experiment in compartments of fast efflux (probably early endosomes) and of slow efflux (probably late endosomes/lysosomes). In our case, the A and B values were 57 and 48%, respectively. Data in Fig. 4 and the calculated AUC values, also showed that, in this case, addition of 20 μM monensin only slightly influenced the intensity of the intracellular fluorescence signal at any of the measured time points. Therefore, it seems that with the NP system, F-oligonucleotides are located predominantly in a non-acidic compartment. This would be advantageous, since in such sequestered locations, oligonucleotides cannot interact with mRNA.

These preliminary experiments suggest that the use of NP allows improved compartmentalization of F-oligonucleotides. Moreover, since no acidification was observed with F-oligonucleotide-loaded NP, these kinds of carrier appear

Table 2

Area under the normalized[a] fluorescence-time curve of the efflux experiments

	monensin	AUC	AUC – mon/ AUC + mon
F-oligonucleotide-loaded NP	–	113.17	
F-oligonucleotide-loaded NP	+	146.58	0.77
Free F-oligonucleotide	–	1057.78	
Free F-oligonucleotide	+	3179.5	0.33

[a]To allow comparison of profiles, the experimental data have been normalized. A value of 100 was attributed to the initial $t = 0$ time point.

to be promising for oligonucleotide delivery to non-sequestered intracellular compartments.

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