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Evaluation of antisense oligonucleotide loaded chitosan nanoparticles; characterization and antisense effect

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Received June 6, 2009, accepted June 19, 2009

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Pharmazie 64: 807–811 (2009) *doi: 10.1691/ph.2009.9193*

The objective of this study was to investigate the effect of different formulation parameters [i.e. molecular weight and concentration of chitosan, concentration of tripolyphosphate (TPP) and use of alginate] on physico chemical and antisense properties of antisense oligonucleotide (AsODN) loaded chitosan nanoparticles (NPs). Preparation methods of phosphodiester (PO) and phosphorothioate (PS) AsODNs-NPs were also compared. AsODN was designed to target the β-galactosidase (β-gal) gene. HeLa cells were used for *in vitro* transfection studies and β-gal was assayed spectrophotometrically. AsODN-NPs obtained were in general positively charged with size between 221.4–525.7 nm depending on formulation. Encapsulation efficiency of NPs depended on the type of backbone of the AsODN. PO-AsODN encapsulation into NPs (78–94%) was less efficient than PS encapsulation (91–98%). The pH of the chitosan solution affected AsODN entrapment. PO-NPs exhibited faster AsODN release than NPs containing PS. In general higher β-gal inhibition was obtained after transfection of AsODN-NPs in cell culture studies. PS-NPs exhibited a higher inhibition effect and the highest (90.71%) inhibition was obtained with formulation PT-2. PS-adsorbed NPs showed an 88% reduction in β-gal. This study can form the basis for forthcoming *in vivo* studies related to AsODN carrier systems that will use chitosan.

1. Introduction

Gene silencing with AsODN has great potential as a novel therapeutic approach to treat diseases. However, the application of this molecule presents serious challenges such as cell penetration, non-specific binding and poor stability. Thus, the use of particulate carriers such as liposomes or nanoparticles may be a more realistic approach to deliver AsODN (Lambert et al. 2001) and, as reported by Fattal et al. (1991), nanoparticles have been shown to be more efficient carrier systems than liposomes due to their better stability. Encapsulation is a popular system for AsODN delivery. Different polymers including polystyrene (Fritz et al. 1997), polyalkylcyanoacrylate (Chavany et al. 1992), poly(lactic acid)-PEG (Emile et al. 1996), PLGA and dendrimers (Hollins et al. 2004, Weyermann et al. 2005), Lambert et al. (2001), have been used in the preparation of nanoparticulate systems for AsODN delivery. Recently Gao et al. (2005), demonstrated the use of chitosan to deliver AsODN efficiently to hepatic cells.

Among the cationic polymers, chitosan is a good candidate for gene transfection because of its biodegradability, biocompatibility and low toxicity. However, very little information is available about AsODN encapsulation into chitosan nanoparticles (Calvo et al. 1998; Gao et al. 2005; Springate et al. 2005).

Many of these studies are about chitosan-AsODN complexes (Calvo et al. 1998; Gao et al. 2005; Springate et al. 2005), Föger et al. (2006), where depolymerized chitosan is complexed with ODN to form solid nanoparticles and the nanocomplexes are used for anti-malarial treatment. These AsODN chitosan nanoparticles exhibit a strong inhibitory effect on parasite growth. Like DNA, AsODNs can be associated efficiently with chitosan nanoparticles. However, Calvo et al. (1998), showed that AsODN was more efficiently associated when the nanoparticles were formed by ionic gelation with TPP. Nafee et al. (2007) prepared flexible chitosan coated PLGA nanoparticles for DNA/RNA delivery and demonstrated that these particles effectively bind the antisense molecules and are taken up into the cells. Recently, Dung et al. (2007) suggested chitosan-TPP nanoparticles as a release system for AsODNs in the oral environment. There has been no detailed study of the effect of formulation parameters on encapsulation and transfection of AsODNs, however, as reported by Nafee et al. (2007), the ability to control the characteristics of the nanoparticle, most importantly the particle size and surface charge, is central in determining the efficiency of transfection.

The aims of this study were to investigate the effect of formulation factors on the nanoparticle characteristics and *in vitro* antisense effect of AsODN-chitosan nanoparticles. 15-mer Phosphodiester (PO) and phosphorothioate (PS) ODN against the β-galactosidase enzyme gene were encapsulated with chitosan polymer using two different concentrations (0.25 and 2.5 mg/ml). TPP and alginate were used for ionic gelation. Nanoparticles were prepared using two different molecular weights of chitosan (low and medium mol wt). Moreover, the pH of the chitosan solution was varied from 3.23 to 5.00.

Table 1: Codes, formulations and properties of phosphodiester (PO) and phosphorothioate (PS) AsODN loaded chitosan nanoparticles (n = 3)

Codes	Chitosan conc. (%)	Alginate conc. (%)	TPP conc. (%)	Encapsulation efficiency (% ± SD)	Particle size (nm ± SD)	Zeta potential (mV ± SD)
S-3		0.050	—	88.39 ± 2.59	338 ± 4.2	12.5 ± 0.7
S-5	0.025		0.025	94.33 ± 1.96	310 ± 7.5	20.4 ± 0.7
S-6		—	0.050	92.11 ± 3.02	364 ± 6.0	23.6 ± 0.7
S-1*				83.85 ± 2.17	426 ± 5.6	28.3 ± 1.4
S-2	0.25		0.25	78.87 ± 2.47	410 ± 7.0	26.6 ± 1.1
S-4		0.25	—	82.99 ± 2.07	525 ± 6.5	17.9 ± 1.2
PT-8**			—	97.34 ± 1.45	297 ± 11.1	10.4 ± 0.9
PT-4	0.025	0.050		96.35 ± 1.77	330 ± 8.0	13.9 ± 0.7
PT-2			0.025	98.03 ± 1.06	221 ± 4.0	20.5 ± 1.6
PT-5			0.050	97.54 ± 1.36	376 ± 6.7	23.7 ± 1.0
PT-1*		—		94.94 ± 4.42	422 ± 6.5	26.9 ± 1.6
PT-3	0.25		0.25	91.49 ± 1.95	426 ± 7.1	26.9 ± 1,6
PT-7**				95.89 ± 1.69	362 ± 7.5	16.2 ± 0.6
PT-6		0.25	—	93.06 ± 2.29	518 ± 7.5	16.7 ± 1.4

* All formulations prepared at pH 5.0 except S-1 (pH 3.5) and PT-1 (pH 3.5)
* PT-7 and PT-8 formulations prepared with low mol wt. chitosan
S coded formulations contain phosphodiester and **PT** coded formulations contain phosphorothioate AsODN
All formulations contain 100 μg of antisense oligonucleotides

2. Investigations, results and discussion

2.1. Characterization of nanoparticles

All the formulations showed an AsODN loading efficiency of over 78.87% (Table 1). The encapsulation efficiency of chitosan nanoparticles increased slightly with the molecular weight of chitosan ($p < 0.001$). As mentioned above, in this study ionic gelation and adsorption methods were used to prepare AsODN-chitosan nanoparticles since both methods are mild and simple. All the AsODN used in the formulations was adsorbed on to the chitosan nanoparticles using the adsorption method.

Table 1 summarizes the results of physical characterization of the AsODN-chitosan nanoparticles. The mean size of nanoparticles varied between 221 and 525 nm depending on the formulation. However, in some formulations, aggregation was observed during the particle size measurement, and, conversely, the nanoparticles had a diameter between 160–200 nm by SEM analysis (data not shown). The differences were not significant between nanoparticles prepared with different chitosan concentrations and preparation methods ($p > 0.05$).

The surface charge of AsODN loaded chitosan nanoparticles in pH 7.4 phosphate buffer ranged from +10 to +28 mV (Table 1). Nanoparticles prepared with a high concentration of chitosan exhibited a high surface charge (+26 to +28 mV), however a lower zeta potential was observed with nanoparticles formed using chitosan at low concentrations (+20 to +23 mV). The use of alginate instead of TPP decreased the surface charges of the nanoparticles. Nanoparticles formed with low molecular weight chitosan also showed a lower surface charge, close to neutral.

AsODNs could be incorporated very efficiently in all nanoparticle formulations. The encapsulation efficiency of the nanoparticles depended on the type of backbone of the AsODN. Phosphodiester encapsulation into chitosan nanoparticles was lower and varied between 78 and 94% (Table 1). PS-loaded nanoparticles displayed the highest encapsulation efficiency with a value of 91 to 98%. The pH value of the chitosan solution affected the encapsulation of AsODNs ($p < 0.05$). When the pH of the chitosan solution was increased from pH 3.2 to pH 5.0, AsODN encapsulation decreased slightly as expected. The effect of chitosan, alginate and TPP concentrations on AsODN encapsulation is not clear, however slightly lower AsODN encapsulation was measured with nanoparticles containing chitosan at a high concentration. When PS and PO encapsulation were compared, the encapsulation efficiency of AsODN into PS-loaded nanoparticles was higher than with PO-loaded nanoparticles ($p < 0.05$).

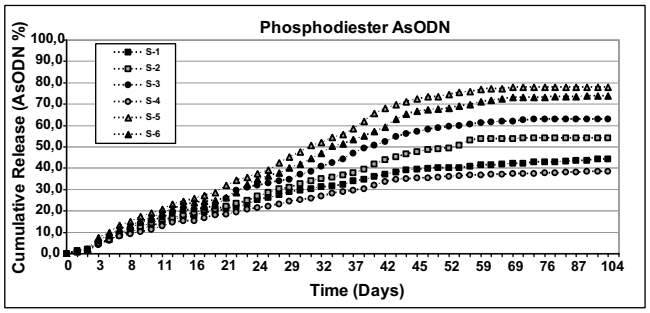

Fig. 1: Release pattern of phosphodiester (PO) loaded chitosan nanoparticles, prepared according to different formulations (n = 3); chitosan concentrations (0.025 and 0.25%), TPP concentrations (0.025, 0.05 and 0.25%) and alginate concentrations (0.05 and 0.25%)

2.2. Release studies

AsODN release was investigated for all nanoparticle formulations and is presented as the fraction of AsODN released into the medium (Figs. 1–3). Within the first few days, the amount of AsODN released was very low [phosphorothioate (PS) loaded nanoparticles 5–10% and phosphodiester (PO) loaded nanoparticles 8–13%], and then the release rate increased. This means that AsODN is incorporated within the chitosan nanoparticle structure and very little AsODN is present at the surface of the nanoparticles. In general, AsODN release from chitosan nanoparticles obeyed zero-order release kinetics. Nanoparticles containing PO and PS exhibited a similar shape of release profile as seen in Figs. 1–3; however the amount of AsODN released was different. PO-loaded nanoparticles exhibited faster AsODN release (Figs. 1 and 2) than PS-containing nanoparticles ($p < 0.05$).

Higher AsODN release was measured from PO-loaded nanoparticles than from PS-nanoparticles (Figs. 1 and 2) and release was not completed during the release studies. Using alginate instead of TPP for chitosan formation led to a significant decrease in the

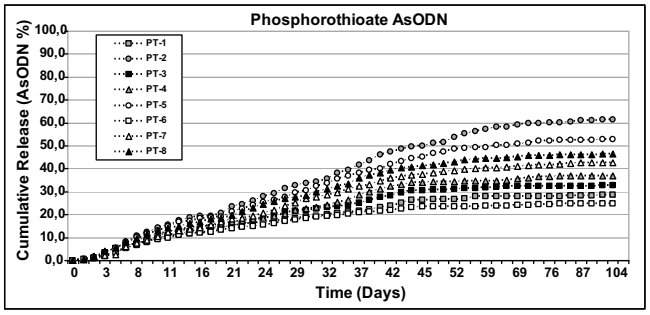

Fig. 2: Release pattern of phosphorothioate (PS) loaded chitosan nanoparticles, prepared according to different formulations (n = 3). Different mol wt (PT 1-6; PT 7,8) and concentrations of chitosan (0.025 and 0.25%), TPP concentrations (0.025, 0.05 and 0.25%) and alginate concentrations (0.05 and 0.25%)

release of AsODN from nanoparticles ($p < 0.001$) (Figs. 1 and 2). This difference in the release patterns of AsODN could be attributed to a reduced AsODN-chitosan interaction with TPP use as reported by Calvo et al. (1997, 1998). Fig. 3 shows AsODN release from nanoparticles prepared by the adsorption method. Among the formulation factors investigated, the molecular weight of chitosan and the pH of preparation did not affect AsODN release ($p > 0.05$).

The stability of AsODN during the release studies was checked by agarose gel electrophoresis. Samples of released AsODN were applied to agarose gel; as seen in the gel photographs (Fig. 4), the released AsODN remained stable during the release experiments.

2.3. In vitro antisense effect of nanoparticles

In general all the nanoparticles containing AsODN showed an antisense effect after *in vitro* transfection to β-Gal expressed HeLa cells, but some of the PS-loaded formulations were more effective in silencing β-gal expression than PO-loaded nanoparticles (Figs. 5 and 6). As seen in Table 2 the reduction of β-gal expression was around 61 – 71% after PO-loaded chitosan nanoparticles were transfected. The highest β-gal inhibition (from 4.77 ng to 0.44 ng) was found using nanoparticles coded PT-2 prepared with 0.025% chitosan, with β-gal expression reduced about 90.71%. As seen in Table 2 the silencing effect of PS-loaded nanoparticles varied from 68 to 90%.

Chitosan nanoparticles prepared by AsODN adsorption on to chitosan-TPP nanoparticles also showed a high silencing effect (88%), close to but significantly different ($p < 0.05$) from the silencing value of formulation PT-2 (Fig. 6). The pH of the nanoparticle preparation medium (S-1/S-2 and PT-1/PT-3) had no effect on the antisense activity of the nanoparticles ($p > 0.05$).

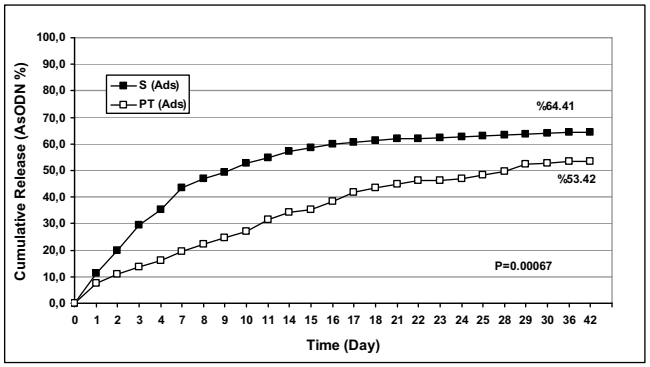

Fig. 3: Release of AsODN from chitosan nanoparticles prepared by adsorption method

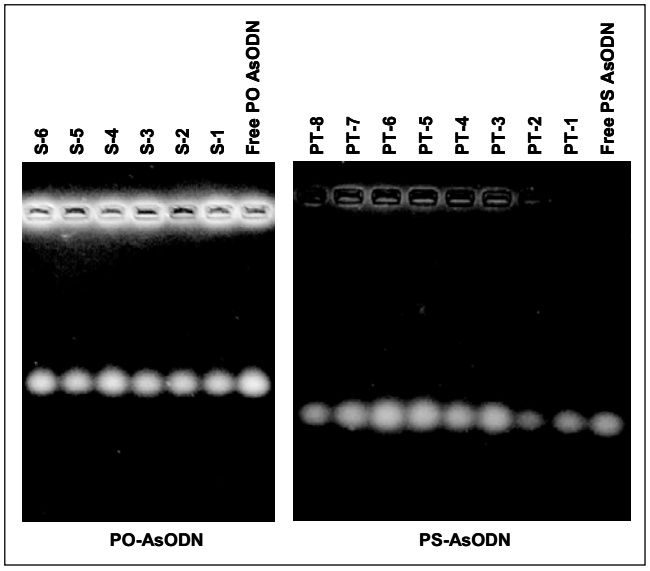

Fig. 4: Agarose gel photographs of released samples of PS and PO-antisense oligonucleotide nanoparticles

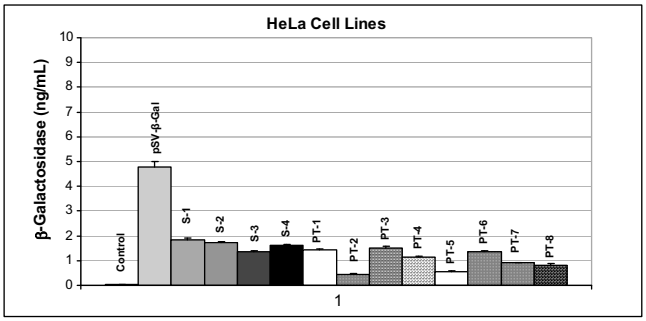

Fig. 5: β-gal inhibition of chitosan nanoparticles prepared by ionic gelation method after 48 hrs of antisense oligonucleotide formulation. S1-S4 PO-AsODN-loaded; PT1-PT8 PS-AsODN-loaded chitosan nanoparticles (n = 4)

When a comparison was made between use of TPP and alginate for preparation, nanoparticles formed with TPP exhibited higher a antisense effect than those prepared with alginate (Table 2). The molecular weight of chitosan also significantly affected the β-Gal inhibition properties of the chitosan nanoparticles (PT-4 and PT-8), $p < 0.001$.

In this study, AsODN-loaded chitosan nanoparticles were prepared using ionic gelation and adsorption methods, since the ionic gelation technique is simple and mild. Cryoprotectant was not added to the nanoparticle formulation, because, as reported

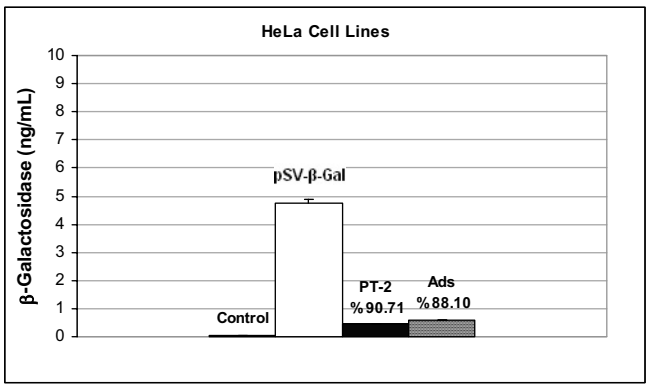

Fig. 6: Comparison of antisense effect of nanoparticles prepared by ionic gelation and adsorption methods (PS-AsODN) (n = 4)

Table 2: Knockdown of β-Gal in HeLa cells with AsODN-loaded chitosan nanoparticles after 48 h of AsODN application (n = 4)

Formulation	Silencing effect (%)
S-1	61.40 ± 0.07
S-2	64.13 ± 0.05
S-3	71.49 ± 0.05
S-4	66.19 ± 0.04
PT-1	70.29 ± 0.06
PT-2	90.71 ± 0.03
PT-3	68.05 ± 0.06
PT-4	76.09 ± 0.03
PT-5	88.62 ± 0.04
PT-6	71.16 ± 0.04
PT-7	80.86 ± 0.02
PT-8	82.83 ± 0.05
Adsorption	88.10 ± 0.02

recently, the TPP content influences the preservation of the integrity and physico chemical properties of chitosan nanoparticles after reconstitution (Fernandez-Urrusuno et al. 1999b). Janes et al. (2001) suggested a chitosan:TPP weight ratio from 3:1 to 6:1 for producing a high yield of stable nanoparticles; however a 1:1 chitosan:TPP weight ratio was used for nanoparticle preparation in our study and stable nanoparticles were obtained (Janes et al. 2001).

The release properties of AsODN-loaded nanoparticles depend to a great extent on the formulation parameters, and formulation variables affecting the *in vitro* characteristics of the nanoparticles were investigated in this study. Modulation of nanoparticle properties could be done easily by using the ionic gelation method as reported earlier (Gan et al. 2005; Gao et al. 2005).

Moreover, among drug carriers, nanoparticles have shown an interesting potential to bind and deliver AsODN in an efficient manner (Fattal et al. 1998) and nanoparticles have a relatively higher cellular uptake compared to microparticles (Bivas-Benita et al. 2004).

Nanoparticles were chosen as the AsODN carrier in this study. The cellular uptake of naked AsODN is generally inefficient and only a small number of ODN molecules actually gain entry to the cell. The cellular uptake mechanism involved depends on factors such as AsODN chemistry, length, conformation and concentration (Akhtar et al. 2000). Free phosphodiester (PO-ODN) and phosphorothioate oligonucleotides (PS-ODN) were used and compared. The size of the nanoparticles varied between 221–525 nm in this study. Chitosan concentration and pH of the chitosan solution were shown to affect the particle size (Table 1). A smaller particle size was obtained with lower molecular weight chitosan; this may be due to decreased viscosity of lower molecular weight chitosan as reported previously by Janes et al. (2001).

High AsODN entrapment was obtained using the ionic gelation method. More efficient AsODN association was found when the nanoparticles were prepared by ionic gelation with TPP than with alginate (Calvo et al. 1998; Janes et al. 2001).

In general encapsulation of PO into the chitosan nanoparticle is lower than encapsulation of PS. It can be said that the chemistry of the antisense oligonucleotides has an effect on the physico chemical properties of the nanoparticles. These results were concordant with those of our previous study of AsODN-chitosan complexes (Enneli et al. 2007).

Moreover, 88% gene knockdown was found with AsODN-adsorbed chitosan nanoparticles, but a higher silencing effect was obtained post-transfection of AsODN encapsulated nanoparticles.

In conclusion, the physicochemical properties (such as size, surface charge and encapsulation), release profiles and *in vitro* antisense effect of AsODN chitosan nanoparticles altered according to formulation parameters. A higher silencing effect was obtained with AsODN chitosan nanoparticles prepared using a low concentration of chitosan.

3. Experimental

3.1. Materials

Phosphorothioate-modified (PS) and phosphodiester (PO) AsODNs (15 mer) were designed to be antisense to a sequence of the β-galactosidase enzyme gene (Fillion et al. 2001). All AsODNs were synthesized by MWG Biotech (Germany) and were of HPLC grade. Low molecular weight chitosan (150 kDa, degree of deacetylation 93%) and medium molecular weight chitosan (400 kDa, degree of deacetylation 87%) were obtained from Fluka (Germany). o-Nitrophenyl-β-D-galactopyranoside (ONPG) and β-galactosidase were obtained from Sigma (USA).

The HeLa cell line was obtained from the American Type Culture Collection (ATCC CLL–2) and cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma, USA) containing 10% fetal bovine serum (FBS), 0.1% antibiotic solution [penicillin (10.000 units/ml), streptomycin (10 mg/ml) and amphotericin B (10 mg/ml)]. Cells were maintained at 37 °C, 5.0% CO_2 in a 95% air humidified atmosphere (Heto-Holten, Denmark). Stable β-galactosidase expressed Hela cells were used in this study.

3.2. Preparation of AsODN-loaded nanoparticles

Nanoparticles were produced by ionic gelation of TPP with chitosan as described previously (Calvo et al. 1997; Fernandez-Urrusuno et al. 1999a). 100 μl of AsODN was added to 10 ml of TPP solution (0.025 – 0.25%) before adding this drop-wise to 10 ml of aqueous chitosan solution under constant stirring. The nanoparticles were then incubated for 30 min. Nanoparticles were separated by centrifugation at 16.000 rpm (5810 R, Eppendorf, Germany) for 10 min. The supernatant was discarded and particles were washed with bidistilled water.

We used two different methods for AsODN association to particles; ionic gelation and adsorption of AsODN on the surface of empty chitosan nanoparticles.

For AsODN adsorption; prepared empty chitosan nanoparticles were dispersed in 100 μl and 2.0 ml AsODN solution was added to nanoparticles and shaken until saturation at room temperature.

3.3. Characterization of nanoparticles

Particle sizes and zeta potential of AsODN loaded chitosan nanoparticles were measured using a Zetasizer 3000 HSA (Malvern Instruments, UK). Nanoparticles were suspended in PBS (pH 7.4) and an average of three separate measurements was recorded.

3.4. Release studies of nanoparticles

AsODN release studies were performed by incubating 10 mg AsODN-loaded nanoparticles in 2 ml PBS pH 7.4 at 37 °C in a shaker. At appropriate intervals, the samples were separated and the amount of released AsODN was determined spectrophotometrically (BioSpec–1601, Shimadzu, Japan) at 260 nm. The mean of three batches was given. After release AsODN integrity was checked by agarose gel electrophoresis.

3.5. Encapsulation efficiency of nanoparticles

AsODN encapsulation efficiency was calculated by measuring the supernatant suspension after the ionic gelation process and the supernatant was spectrophotometrically analyzed at 260 nm for AsODN concentration.

3.6. Cell culture

HeLa cells were seeded at a density of 5.0×10^4 cells/well in 24-well plates with 1 mL of complete medium (DMEM containing 10% FBS) and incubated for 24 h prior to transfection. Transfections were performed on cells at approximately 70% confluence. Before transfection, the complete medium was removed and DMEM without serum was added to the cells. The naked AsODN and nanoparticles (containing 2 μg of AsODN) were diluted in DMEM and then were added to the cells. After incubation at 37 °C for 6 h, DMEM containing 10 % serum was added to the wells and then the medium containing free complexes was removed.

The cells were rinsed twice with PBS, and then 200 µl of Triton X-100/lysis buffer was added to each well. Two freezing and thawing cycles were used for lysis of all cells, 25 µl of cell lysate was pipetted into wells of a 96-well plate, and then 135 µl of Buffer A/β-mercaptoethanol solution was added to each well, and after waiting for 15 min at 37 °C. 50 µl of ONPG solution was added and incubated at 37 °C for 19 h. When a faint yellow color appeared, 90 µl of Na_2CO_3 solution (1 M) was added to each well to stop the reaction. The absorbances of the samples were read at 420 nm with a UV spectrophotometer (Shimadzu UV Biospec–1601, Japan).

3.7. Statistical analysis

All values are the means of three experiments ± SD. Statistical data analysis was performed using Student's t-test of variance with $p < 0.05$ as the minimum level for significance.

Acknowledgement: The authors are grateful for financial support of Marmara University Scientific Research Projects Association (BAPKO, Project no: SAĞ-YLS 270306-0034).

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