Aptamer-Conjugated Calcium Phosphate Nanoparticles for Reducing Diabetes Risk via Retinol Binding Protein 4 Inhibition

Raheleh Torabi PhD a, Hedayatollah Ghourchian PhD a,b,*, Massoud Amanlou PharmD, PhD c,b, Parvin Pasalar PhD d

a Laboratory of Microanalysis, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
b Nanobiomedicine Center of Excellence, Nanoscience and Nanotechnology Research Center, University of Tehran, Tehran, Iran
c Department of Medicinal Chemistry, Faculty of Pharmacy and Medicinal Plants Research Center, Tehran University of Medical Sciences, Tehran, Iran
d Endocrinology and Metabolism Molecular Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

Objectives: Inhibition of the binding of retinol to its carrier, retinol binding protein 4, is a new strategy for treating type 2 diabetes; for this purpose, we have provided an aptamer-functionalized multishell calcium phosphate nanoparticle.

Methods: First, calcium phosphate nanoparticles were synthesized and conjugated to the aptamer. The cytotoxicity of nanoparticles releases the process of aptamer from nanoparticles and their inhibition function of binding retinol to retinol binding protein 4.

Results: After synthesizing and characterizing the multishell calcium phosphate nanoparticles and observing the noncytotoxicity of conjugate, the optimum time (48 hours) and the pH (7.4) for releasing the aptamer from the nanoparticles was determined. The half-maximum inhibitory concentration (IC50) value for inhibition of retinol binding to retinol binding protein 4 was 210 femtomolar (fmol).

Conclusions: The results revealed that the aptamer could prevent connection between retinol and retinol binding protein 4 at a very low IC50 value (210 fmol) compared to other reported inhibitors. It seems that this aptamer could be used as an efficient candidate not only for decreasing the insulin resistance in type 2 diabetes, but also for inhibiting the other retinol binding protein 4-related diseases.

Can J Diabetes 41 (2017) 305–311
Contents lists available at ScienceDirect
Canadian Journal of Diabetes
journal homepage:
www.canadianjournalofdiabetes.com

A R T I C L E  I N F O
Article history:
Received 24 August 2016
Received in revised form 11 October 2016
Accepted 1 November 2016

Keywords:
Aptamer
Calcium phosphate nanoparticles
Inhibitory concentration
Retinol binding protein 4
Type 2 diabetes

A B S T R A C T
Objectives: Inhibition of the binding of retinol to its carrier, retinol binding protein 4, is a new strategy for treating type 2 diabetes; for this purpose, we have provided an aptamer-functionalized multishell calcium phosphate nanoparticle.

Methods: First, calcium phosphate nanoparticles were synthesized and conjugated to the aptamer. The cytotoxicity of nanoparticles releases the process of aptamer from nanoparticles and their inhibition function of binding retinol to retinol binding protein 4.

Results: After synthesizing and characterizing the multishell calcium phosphate nanoparticles and observing the noncytotoxicity of conjugate, the optimum time (48 hours) and the pH (7.4) for releasing the aptamer from the nanoparticles was determined. The half-maximum inhibitory concentration (IC50) value for inhibition of retinol binding to retinol binding protein 4 was 210 femtomolar (fmol).

Conclusions: The results revealed that the aptamer could prevent connection between retinol and retinol binding protein 4 at a very low IC50 value (210 fmol) compared to other reported inhibitors. It seems that this aptamer could be used as an efficient candidate not only for decreasing the insulin resistance in type 2 diabetes, but also for inhibiting the other retinol binding protein 4-related diseases.

RÉSUMÉ
Objectifs : L’inhibition de la liaison du rétinol à son transporteur, la protéine 4 de liaison au rétinol, est une nouvelle stratégie de traitement du diabète de type 2. À cet effet, nous avons fourni une nanoparticule multicoquille de phosphate de calcium fonctionnalisée par l’aptamère.

Méthodes : D’abord, les nanoparticules de phosphate de calcium ont été synthétisées et conjuguées à l’aptamère. La cytotoxicité des nanoparticules déclenche le processus de libération de l’aptamère des nanoparticules et leur fonction d’inhibition de la liaison du rétinol à la protéine 4 de liaison au rétinol.

Résultats : Aprés la synthèse et la caractérisation des nanoparticules multicoquilles de phosphate de calcium et l’observation de la non-cytotoxicité du conjugué, nous avons déterminé la durée optimale (48 heures) et le pH (7,4) pour libérer l’aptamère des nanoparticules. La valeur de concentration inhibitrice semi-maximum (IC50) pour l’inhibition de la liaison du rétinol à la protéine 4 de liaison au rétinol était de 210 femtomoles (fmol).

Conclusions : Les résultats ont révélé que l’aptamère pourrait prévenir la liaison entre le rétinol et la protéine 4 de liaison au rétinol à une très faible valeur IC50 (210 fmol) comparativement aux autres inhibiteurs rapportés. Il semble que cet aptamère pourrait être utilisé comme un candidat efficace non seulement pour diminuer l’insulinorésistance lors de diabète de type 2, mais également pour inhiber les autres maladies liées à la protéine 4 de liaison au rétinol.

Mots clés :
Aptamer
Nanoparticules de phosphate de calcium
Concentration inhibitrice
Protéine de liaison au rétinol4
Diabète de type 2

© 2017 Canadian Diabetes Association.

* Address for correspondence: Hedayatollah Ghourchian, PhD, Institute of Biochemistry & Biophysics, University of Tehran, Enghelab Street, Tehran, Iran.
E-mail address: ghourchian@ut.ac.ir

1499-2671 © 2017 Canadian Diabetes Association.
The Canadian Diabetes Association is the registered owner of the name Diabetes Canada.
http://dx.doi.org/10.1016/j.jcjd.2016.11.001
Introduction

Type 2 diabetes has become a major challenge for public health worldwide. Aging, urbanization, increasing rates of obesity, physical inactivity and modern lifestyles are the factors that contribute to increased insulin resistance and the prevalence of this chronic disease (1). One of the reliable markers for the prediction of insulin resistance is retinol-binding protein 4 (RBP4). In recent years, it has been revealed that RBP4 is secreted by adipose tissue. Therefore, RBP4 is related to adiposity, insulin resistance and type 2 diabetes (2). The molecular mechanism of RBP4 in inducing insulin resistance is controversial and complex. However, studies have shown that increasing RBP4 concentration in serum disrupts the insulin pathways in glucose metabolism, and this leads to hyperglycemia and, consequently, to type 2 diabetes (3). But it has also been shown that elevation of RBP4 induces adipose tissue inflammation and impairs glucose tolerance and insulin sensitivity, which cause insulin resistance (4).

RBP4 is the principal carrier of retinol in the human plasma, and it forms a complex with transthyretin (TTR), a homotetrameric thyroxin transport protein. This complex is thought to prevent glomerular filtration of RBP4. Binding retinol to RBP4 induces conformational changes that cause greater affinity between the retinol-RBP4 complex and TTR (retinol-RBP4-TTR) than RBP4 and TTR (RBP4-TTR) (5). Changing RBP4 activity (the ability of RBP4 to bind with retinol and TTR), altering the structural stability of RBP4 in tissues or the half-life in circulation could be targets for the treatment of insulin resistance (6). It seems that the inhibition of binding retinol to RBP4 can decrease the affinity of RBP4 to TTR, which may result in renal clearance of RBP4 and the lowering of serum RBP4 (7). Many synthetic retinoid compounds have been reported as retinol antagonists, which disrupt the interaction between RBP4 and its binding partner (TTR), and that leads to lower serum levels of RBP4 (8,9).

Aptamers, a new generation of antagonists, are a special class of nucleic acid molecules (single-stranded DNA or RNA oligonucleotides) that can bind to their targets with high affinity and specificity due to their tertiary structures (10). Since 1990, aptamers have been used in medicine as therapeutics, for example, pegaptanib sodium injection (Macugen), which targets age-related macular degeneration. Many aptamers, now in differing stages of clinical development, have been discovered to affect a variety of diseases. Thus, in the future, we can expect more therapeutic aptamers to be available on the market for clinical applications (10–13).

Recently, a 76-mer single-stranded DNA aptamer was reported; it binds specifically to human RBP4 protein (RBP4 binding aptamer) (RBA) (14). A molecular dynamic simulation study of RBP4-RBA interaction, which was also done by this research group, showed that RBA binding to RBP4 probably inhibits RBP4 interactions with retinol by detection of RBP4 active pocket residues (15). It seemed that in the case of proving the inhibition effect of RBA on binding retinol to RBP4, we could introduce a new therapeutic candidate for inhibition of retinol-RBP4 complex formation to cause disruption in RBP4 binding to TTR, which drives renal clearance of RBP4, lowering serum levels of RBP4 and, consequently, decreasing insulin resistance in persons with type 2 diabetes.

The specific challenges of therapeutic aptamers generally include nuclease-mediated degradation, rapid renal filtration and structural stability. Currently, to increase the structural stability and improve the half-life of aptamers, they are conjugated to nanoparticles extensively (16). One of the carriers of nucleic acids is calcium phosphate nanoparticles (CP-NPs), which are biocompatible and biodegradable and also are not subject to microbiologic degradation as are organic and polymeric carrier systems. The affinity of calcium of CP-NPs with the phosphate groups in nucleic acids is probably due to the electrostatic interaction between the negative charges of DNA and the positive charges of calcium ions. It has also been demonstrated that DNA can be protected against nuclease attacks if it is enclosed within CP-NPs with an additional shell of calcium phosphate (17). CP-NPs, with their fast degradation rate in cells, are appropriate for applications in drug delivery (18).

In the present study, the synthesis of RBA-conjugated multishell CP-NPs was described. The procedure was so mild that the function and structure of RBA were conserved. In addition, these prepared nanoparticles could inhibit the binding of retinol to RBP4 without showing toxicity, so they could be suggested as efficient candidates for decreasing insulin resistance in patients with diabetes.

Methods

Materials and reagents

Human RBP4 (a full-length protein) was provided by Abcam (Cambridge, Massachusetts, United States). A 76-mer RBA was purchased from Bioneer (Daedeok-gu, Daejeon, Republic of Korea). Calcium nitrate (Ca(NO₃)₂), aluminum chloride (AlCl₃), ammonium phosphate dibasic ([NH₄]₂HPO₄), retinol (R7623), Dulbecco/Vogt modified Eagle minimal essential medium (DMEM), fetal bovine serum (FBS) and thiazolyl blue tetrazolium bromide (MTT) were prepared (Sigma, St. Louis, Missouri, United States). A fibroblast NIH-3T3 cell line was obtained from the Pasteur Institute (Tehran, Iran).

Calcium phosphate nanoparticle synthesis

CP-NPs were prepared according to the literature, with a slight modification (19). First, to control the size growth of CP-NPs, calcium was partially substituted by aluminum by adding AlCl₃ (2 mM) to the Ca(NO₃)₂ solutions (30 mM, pH 9). Briefly, aqueous solutions of Ca(NO₃)₂ were pumped (flow rate: 34 mL/min) into a vessel containing (NH₄)₂HPO₄ (3.74 mM, pH 9) at room temperature while stirrung. After 30 seconds, 100 μL of the nanoparticles (pH 8) were taken up with a syringe and mixed rapidly with a solution of RBA (100 μL of 60 ng/mL) at room temperature to result in the CP-NPs’ being coated with RBA (RBA/CP).

To change RBA/CP into multishell nanoparticles, the following steps were carried out. First, 0.5 mL of calcium nitrate (30 mM, pH 9) were added to the RBA/CP dispersion, followed by the addition of 0.5 mL (NH₄)₂HPO₄ (3.74 mM, pH 9). This process led to the deposition of calcium phosphate on the surface of the RBA/CP. The resulting CP/RBA/CP colloidal solution was stabilized by adding 100 μL of 60 ng/mL of RBA solution to form RBA/CP/RBA/CP nanoparticles. The final nanoparticles were centrifuged at 9000 rpm for 10 minutes to collect the nanoparticles and remove the unconjugated RBA in the supernatant. This washing step was repeated at least 3 times (20). The final CP-NP conjugates were stable and could be stored at 6°C. The size and zeta potential of the RBA/CP/RBA/CP nanoparticles were measured by dynamic light scattering (90 plus; Brookhaven National Laboratory, Upton, New York, United States).

RBA release

RBP4 was synthesized by hepatocytes and adipocytes and then secreted into the blood (21), so cells and blood circulation can be considered the main targets for releasing RBA to bind RBP4 and inhibit its function. The experiment was carried out at 2 different pHs: 7.4 and 5.0. The pH 7.4 reflects the physiologic pH of blood, and the pH 5.0 indicates the pH of the endosome or skin (22,23). The release of RBA was conducted by dispersing 100 μL of conjugates (RBA/CP/RBA/CP) in 0.8 mL PBS by gentle shaking (20 rpm) at 37°C (20 cycles per minute). After various time intervals (1, 2,
4, 6, 12, 24, 48 and 72 hours), the supernatants were collected by centrifugation at 5000 rpm (7000×g) for 10 minutes. The RBA released in the supernatant was measured by a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, Delaware, United States) at 260 nm. The RBA concentration was calculated automatically based on the following equation: 1 OD_{260} unit = 33 μg/mL RBA (24). To evaluate the dissolution process of the nanoparticles, at time intervals of 24 hours, their particle sizes and surface zeta potentials were measured by dynamic light scattering.

Cytotoxicity of nanoparticles

To study the cytotoxicity of RBA, CP-NPs and RBA/CP/RBA/CP in normal cells, the fibroblast NIH-3T3 cell line was established and cultured according to the supplier’s descriptions. Briefly, the cells were cultured in DMEM medium supplemented with 10% FBS in a humid atmosphere at 37°C and 5% CO2. The cells were treated with free RBA, CP-NPs and RBA released from RBA/CP/CP/CP. Then the samples were incubated in PBS (pH 7.4) at 37°C for 24 hours on a rocker and the MTT assay was applied to screen the cell proliferations (25).

Efficiency of the released RBA in capturing RBP4

To determine the efficient dosage of the released RBA in capturing RBP4, the concentration of RBP4 was prepared and aliquoted into an Eppendorf tube. Then a serial dilution of the released RBA was placed into tubes and incubated for 2 hours. After centrifugation at 9000 rpm for 10 minutes, the free RBP4 concentration in the supernatant was determined by chemiluminescence. First, RBA was immobilized in a 96-well plate as the capturing element, and then the free RBP4, as the antigen, was added to the plate. In the third step, the RBA-RBP4 complex was incubated with the luminol-conjugated anti-RBP4 polyclonal antibody to form an immunosandwich. After washing the untreated antibodies in the presence of H2O2 and catalyst, the chemiluminescence signal, which is proportional to free RBP4 concentration, was recorded (26) and used for calculation of the half-maximal inhibitory concentration (IC50), which was defined as the concentration of RBA that is able to decrease the free RBP4 concentration to 50%.

Inhibition of retinol binding to RBP4

The inhibition effect of RBA on RBP4 binding to retinol was studied by the fluorimetric method with monitoring of emissions after excitation at 280 and 330 nm (Varian Cary Eclipse fluorescence spectrophotometer, Santa Clara, California, United States). First, a 0.5 μM solution of RBP4 in PBS (pH 8.0) and a 250 μM solution of retinol in DMSO were prepared. The same amounts of RBP4 and retinol (0.5 μM) were aliquoted into Eppendorf tubes. Serial dilutions of RBA released from CP-NPs were added to them and incubated at room temperature with mild shaking for 1 hour. Under dim lighting, the retinol solution (final concentration of 0.5 μM) was added to each tube and incubated at room temperature with constant stirring for 5 minutes. Finally, the fluorescence intensity of each tube was measured so as to ascertain the quantity of retinol binding to RBP4. Afterwards, a calibration curve was prepared by plotting the measured values and then, based on these data, the IC50 was calculated. The IC50 was defined as the concentration of RBA that is able to decrease the retinol-RBP4 binding to 50%.

The method of IC50 calculation

To calculate IC50, BioDataFit, an online data-fitting program (http://www.changbioscience.com/stat/ec50.html), designed specifically for biologic assays, was applied based on 4 parameter logistic regression, a useful method of determining dose responses and receptor-ligand binding assays.

Results

Calcium phosphate nanoparticle characterization

The size and zeta-potential changes in CP-NPs via differing coatings are summarized in Table 1. Increasing the sizes and surface zeta potentials during the addition of coating can confirm this process. The arbitrary sizes of nanoparticles can be controlled by the parameters, such as reactant concentrations and incubation times. Zeta potential is a qualitative measure that demonstrates the altering layer of the multishell nanoparticles. As shown in the first row of Table 1, CP-NPs showed a strongly positive charge (+22), which can be explained by the fact that the nanoparticles’ positive charge depends on the ions (such as Mg2+, Sr2+, Ba2+, Zn2+, Fe2+, Mn2+ and Al3+) substituted on the CP-NPs’ surface. But the nanoparticles’ charges tend to be negative when they are covered by DNA molecules (RBA/CP) (19). However, the charge of the nanoparticles becomes only slightly positive when covered by CP. With the addition of the second CP layer on RBA/CP (CP/RBA/CP), the zeta potential becomes slightly positive. But eventually, the fourth layer of NPs (RBA/CP/RBA/CP) shifts the zeta potential to negative due to the negative charges on the phosphate backbone.

RBA release from NPs

For comparison between the release of RBA from CP-NPs into a semblance of blood circulation (pH 7.4) and cellular endosome (pH 5), an experiment was carried out. Figure 1A shows the resulting changes in CP-NPs at pH 7.4. As seen, in the first 24 hours, by releasing the RBA, its concentration increased gradually (solid line). On the other hand, monitoring the nanoparticle size confirmed that during the first 24 hours, the nanoparticle size decreased from ~90 nm to ~60 nm (dashed line). In addition, during the first 24 hours, by removing the RBA from the outer layer (RBA), the zeta potential increased from ~1 to ~5 mV. This change indicates the dissolution of the fourth layer (RBA) and exposure of the third layer

<table>
<thead>
<tr>
<th>Type of nanoparticle</th>
<th>Layers scheme</th>
<th>Size (nm)</th>
<th>Zeta potential (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td></td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>RBA/CP</td>
<td></td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>CP/RBA/CP</td>
<td></td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>RBA/CP/RBA/CP</td>
<td></td>
<td>87</td>
<td>−1</td>
</tr>
</tbody>
</table>

Table 1
Size and zeta potential of CP-NPs with different coatings
Changes of absorption at 260 nm in the second 24 hours shows that the release rate sped up and reached its maximum level; then, during the third 24 hours, the RBA concentration remained constant (solid line). On the other hand, measuring particle size during these second 24 hours showed that the size of nanoparticles decreased rapidly (dashed line) so that the zeta potential decreased from +5 to 0 mV. This could be a sign that during this period, the third layer (CP) was also dissolved, and the remaining second layer (RBA) was exposed. These experimental results showed that at pH 7.4, the RBA-CP-NPs complex is dissociated layer by layer during 48 hours.

On the other hand, the release of RBA from CP-NPs at pH 5.0 revealed that in the first 24 hours, RBA released rapidly and reached its maximum value (Figure 1B, solid line). Changes in absorption at 260 nm in the second 24 hours showed that the release rate declined and reached its minimum level and then, during the third 24 hours, the RBA concentration remained constant (solid line). The rapid declension of released RBA at this pH could be due to the fact that at this acidic pH, the released RBA is changed into a coiling form and loses its functional folding structure (27). Measuring the particle size over time has also shown that at pH 5.0, nanoparticles decompose rapidly (Figure 1B, dashed line).

In vitro cytotoxicity testing

An MTT assay was employed to identify the cytotoxic effects of free RBA, CP-NPs and the released RBA from RBA/CP/RBA/CP on the proliferation of cells. The result indicates the noncytotoxic effect of free RBA, CP-NPs and released RBA on the proliferation of cells (data not shown).

Evaluation of RBA-RBP4 binding

To determine the efficient dosage of the released RBA in capturing RBP4, according to the procedure explained in the experimental section, the free RBP4 concentration was determined by chemiluminescence. As shown in Figure 2, by increasing RBA concentration, the chemiluminescence intensity declined, indicating RBP4 capturing by RBA and the decreasing of free RBP4 concentration. The calibration curve was plotted, and the IC50 for RBA was obtained; it was 1.16±0.43 ng/mL (41.57±15.41 femtomolar [fM]).

Inhibition of retinol binding to RBA-RBP4

Of the 4 tryptophan (Trp) residues in RBP4, 2 are buried (24 and 105) and 2 are exposed (67 and 91), which are available as intrinsic probes for monitoring their interactions. In addition, RBP4, after binding to retinol, becomes more fluorescent. This offers a variety of possible uses of fluorescence as a tool in the study of the inhibition effect of RBA on the binding of retinol to RBP4 (28). As shown in Figure 3, A, by exciting RBP4 at 280 nm (solid line), a typical Trp fluorescence emission is observed at 335 nm. By formation of the RBP4-retinol complex (dotted line), Trp fluorescence emission is drastically diminished, while a second fluorescence emission peak is produced at 470 nm due to the Förster resonance energy transfer event between RBP4 and retinol. This peak emanates simultaneously from bound retinol and increases as a function of retinol concentration. By formation of the RBA-RBP4 complex (dashed line), the emission peak of RBP4 undergoes a relative decrease in intensity and a shift to 330 nm. The change in intensity and the blue shift (5 nm) in Trp fluorescence confirms that RBA is attached to RBP4 and covers the Trp residue; consequently, the accessibility of Trp to the bulk solvent is decreased. By adding RBA and retinol, respectively, to RBP4, the emission peak at 330 nm (dashed dotted line) shows weaker intensity than the RBA-RBP4 complex (dashed line); however, the intensity is still higher than that of the RBP4-retinol.
complex (dotted line). In contrast, the emission intensity of this mixture at 470 nm is lower than that of the RBP4-retinol complex, which indicates the declining of the retinol concentration bound to RBP4. Therefore, one may conclude that presence of RBA may be led to inhibit retinol binding to RBP4. In other words, we could say that in this stage, 2 complexes (RBA-RBP4 and RBP4-retinol) are present in the solution.

Retinol itself shows an emission peak at 420 nm and excitation at 330 nm, which shifts to 480 nm due to binding to RBP4. To assay and quantify the inhibition effect of RBA on RBP4-retinol binding, in an independent experiment, in the presence of serial dilution of RBA, retinol bound to RBP4 was excited at 330 nm, and the emission peak was measured at 480 nm. In Figure 3B, the fluorescence emission intensity at 480 nm declined by increasing RBA concentration, which confirms the inhibition effect of RBA on binding retinol to RBP4. Based on the calibration curve, which was plotted according to these data, the IC\textsubscript{50} for RBA was determined to be 5.2008±0.6216 ng/M (210.88±25.2 fm) (Figure 3C).

Discussion

The chemically synthesized antagonists of RBP4 that compete with retinol for binding to RBP4 (2) which induce conformational changes in RBP4. They also inhibit the formation of retinol-RBP4-TTR complex, which leads to lower serum RBP4 through rapid renal clearance of RBP4 and may improve insulin sensitivity to minimize the risk for type 2 diabetes (4,7,29). In contrast, aptamers, as the new class of antagonists, can inhibit and modulate the function of their target proteins via binding to the target. They can effect physiologic processes to treat pathologies (30). This makes them ideal inhibitors as therapeutic reagents. They simply produce and modify chemically. Because of their small size, aptamers are not directly recognized by the immune system (31).

According to this evidence, in the present study, an aptamer acting against RBP4 (abbreviated as RBA) was used to inhibit retinol-RBP4 binding (14). For increasing serum stability and circulating half-life of RBAs (32,33), RBAs were conjugated to CP-NPs. Characterization of CP-NPs showed that, because of their surface positive charge, aptamers easily conjugated with them in desired sizes. These multishell CP-NPs are noncytotoxic and have acceptable stability. Controlling the RBA-releasing process in vitro is a simple method in which it is possible to estimate the in vivo behaviour of drug-carrier nanoparticles. This provides useful information about the sustainability and efficiency of the nanoparticles along with information about the quantity and rate of RBA released over time. These parameters reflect the physical and chemical parameters of CP-NPs, such as solubility, particle size and active pharmaceutical ingredients (34). From the experimental results obtained during the release process, one may conclude that the layer-by-layer dissociation of CP-NPs at pH 7.4 during 48 hours is similar to the surface erosion mechanism. Rapid decomposition of nanoparticles at pH 5.0 is also close to the characteristic of the bulk erosion mechanism (35). Considering all the aspects, it seems that in order to achieve a regular release rate in the long term, the physiologic pH (7.4) is preferred. Also, in considering the erosion mechanism of these multilayered nanoparticles, it seems that they are suitable for applying in serum-like environments. However, in acidic conditions, they need a more refractory additional layer such as chitosan for greater protection (36). We have also shown that released RBAs conserve their activity to bind RBP4, which confirms the protective effect of CP-NPs.

It is important to note that our findings showed that RBA has much lower IC\textsubscript{50} (210 fm) than other reported inhibitors. The synthetic retinoid (fenretinide, an RBP4 antagonist) increases urinary RBP4 excretion, reduces serum RBP4 levels, improves insulin action in obese mice, and may also have therapeutic effects in type 2 diabetes. The IC\textsubscript{50} for fenretinide was determined to be 56 nM (37). Administration of all-transretinoic acid (another RBP4 antagonist) decreases serum RBP4 (IC\textsubscript{50}=1.2 μM). It suppresses insulin resistance in mice with diabetes. It improves insulin sensitivity and has an antidiabetic influence that could be considered in the treatment of type 2 diabetes (38). Among the RBP4 inhibitors, the best reported IC\textsubscript{50} was for a nonretinoid RBP4 antagonist called A1120, which has an IC\textsubscript{50} of 14.8 nM (8,9). It seems that RBA acts as a competitive inhibitor with differing mechanisms due to its long sequence, which could block the binding site of the retinol in RBP4. In other words, the RBA may block the entry gateway of an internal cavity of RBP4-bound retinol (Figure 4).
Conclusions

Considering the noncytotoxicity of aptamer molecules conjugated to CP-NPs and also their conservative role in the structure and function of RBA, it seems that this conjugate is efficient for use in vivo applications. On the other hand, the ability of RBA to inhibit the retinol binding to RBP4 encouraged us to introduce this conjugate as an efficient candidate for controlling the risks of type 2 diabetes by decreasing insulin resistance.

According to these results, RBA can inhibit binding of retinol to RBP4. RBP4 without retinol has low affinity to TTR. This reduces the RBP4-TTR complex formation. Free RBP4 is rapidly cleared from kidney that cause lower serum levels of RBP4. As a future perspective, we could expect that the RBA-CP-NPs complex has the potential to disrupt the formation of the retinol-RBP4-TTR complex. In the future, it would be interesting to use these conjugates in living animals and follow its direct effect on insulin resistance and type 2 diabetes. In view of other possible clinical applications, RBP4 also seems to be correlated with cardiometabolic markers in inflammatory chronic diseases, including obesity, metabolic syndrome and cardiovascular diseases (39). Investigation of the inhibitory function of RBA in these diseases may also help to extend the application of RBA in therapeutics.

Acknowledgments

Financial support provided by the Research Council of the University of Tehran (Grant No. 1395) and Tehran University of Medical Sciences (Grant No. 94-03-33-30236) are gratefully appreciated.

References


24. Gajjar CR, King MW. Resorbable fiber-forming polymers for biotextile applica-


