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Retinoic Acid-Oligomeric Chitosan Micelles as Novel Gene Delivery Carrier; *in Vitro* Transfection Study

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Amphiphilic cationic micelles have recently attracted much attention as non-viral vector. In the current study the retinoic acid-g-chitosan (RA-chitosan) micelles have been used as novel gene carriers. Uptake of micelles, condensation ability and transfection efficiency of micelle–DNA complexes (miceplexes) in the presence or absence of serum had been investigated on Hela and HepG2 cell lines. Uptake of micelles in Hela cells was faster and more effective than HepG2. After 1.5 hours, micelles were up-taken by Hela cells while no uptake was observed in HepG2 cells. Miceplexes showed lower transfection efficiency in both Hela and HepG2 cells grown in the serum free medium than chitosan polyplexes, with 7.25×10^5 versus 1.47×10^5 RLU.mg-1 and 7.76×10^5 versus 2.16×10^5 RLU.mg-1, respectively. Lower transfection efficiency of miceplexes could be attributed to their stronger complexation. Transfection in the presence of serum increased in both of the cell lines. These properties all together, make RA-chitosan micelles a potential gene carrier for active gene delivery into cells, particularly for transfection in the presence of serum.

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Introduction

The combination of two or more therapeutic approaches with different mechanisms is a promising strategy for effective treatments of cancers. Co-delivery of anticancer drugs/genes not only can decrease the side effects but also overcomes drug resistance [1-3].

Polymeric micelles are one of the best carriers for co-delivery of gene and drug. Recently, several studies have been published on co-delivery of anticancer drugs such as taxol and doxorubicin with pDNAs and siRNAs[2-4]. The micelles have a core–shell structure with an internal core of hydrophobic segments which is useful for loading of hydrophobic drugs, surrounded by hydrophilic segment which is applicable for loading of nucleic acids [2, 4, 5]. They also have a long circulation time, due to the steric hindrance caused by the presence of the hydrophilic shell. As a result, they can target the encapsulated drug to specific tissues, through either passive or active mechanisms [6]. They can also overcome drug resistance that develops in multidrug resistant (MDR) cells [7].

There are various methods to deliver the gene of interest, which can be categorized as viral and non-viral methods. While viral vectors have a high transduction rate, their application has been restricted because of acute immune response, possible infections, probability of carcinogenicity, and lack of control release and low capacity of vector for transformed genes. On the other hand, non-viral vectors are safer and more biocompatible but are restricted by low transfection [8]. However, so many gene therapy studies are under clinical trial with 66.5% targets for cancer therapy [3].

Chitosan, structure component of shellfish and insects, and cell wall of bacteria and mushrooms, and the most ample natural polymer after cellulose, has been used more than 20 years as a non-viral vector, because of its positive charge, biocompatibility and biodegradability [9]. It also has a long history in polymeric micelles as drug carrier [10].

All-trans retinoic acid (ATRA), the biological active form of retinol (vitamin A), plays a critical role in the induction of cell differentiation and the arrest of cell proliferation which is useful for stem cell engineering and cancer therapy. Currently, ATRA is effectively used in differentiation therapy for acute myelogeneousleukemia[11-13].

In this paper, we have optimized the cationic micelles based on all trans- retinoic acid-g-chitosan (RA-chitosan) copolymer for gene delivery. In our previous study, we have produced RA-chitosan micelles and shown that it can be a carrier for anticancer drugs [10]. In the current study, uptake of RA-chitosan micelles and capability of these micelles for loading and delivery of pDNA have been investigated.

Materials and methods

Materials

Chitosan was supplied by Yuhuan Marine Biochemistry Co.,Ltd., Zhejiang, China. pGl3 luciferase plasmid and steady-Glo luciferase assay kit were supplied by Promega. All trans- retinoic acid and other chemical compounds were purchased from Sigma–Aldrich and were used without any purification.

Preparation of RA-chitosan micelles:

RA-chitosan micelle preparation has been explained elsewhere [10]. Briefly, To prepare the micelles, RA–chitosan with degree of substitution of 18.78 ± 0.86 was suspended in water or PBS buffer at 1 $mg.ml^{-1}$, followed by sonication using probe type sonicator (Sonopuls HD 3200, Bandeline, Germany) at 60 W for 2 min (2 s pulse on and 2 s pulse off). Sonication was repeated three times to obtain optically clear solution. Size, zeta and CAC of RA-chitosan micelles were 142.14 ± 5.06, 27.25 ± 6.31 and 1.3 × 10−2 respectively.

Uptake study

To study cellular uptake of nanoparticles by the fluorescence microscopy (Nikon Eclipse E200, Japon), the cells were cultured in 6-well plastic dishes containing coverslips for 24 h. RA-chitosan micelles were added to the cell culture media at a particle concentration of 100 $\mu g.ml^{-1}$. After 1.5 and 5 h of incubation at 37 °C, the cells were washed six times with PBS. The coverslips were put on slides and viewed by fluorescence microscopy. ATRA (10 $\mu g.ml^{-1}$) in 1 percent DMSO was used as a positive control [14].

Preparation of miceplexes

The incorporation of DNA within the micelles was performed through electrostatic interactions between DNA and RA-chitosan micelle. This simply involved adding the aqueous solution of the DNA at the different concentration to the micelle

with the constant concentration in PBS at pH 6, followed by 15 min incubation in r.t., prior to use.

Gel retardation assay

Samples containing 0.165 µg DNA either alone or complex with oligochitosan (OCH) or RA-chitosan micelles at desired charge ratio (CR), were mixed with loading buffer and loaded onto 0.8% agarose gel containing ethidium bromide (0.2 $\mu g.ml^{-1}$). Samples were run in TAE running buffer, using a horizontal gel electrophoresis apparatus. The gel was exposed to an electric field (80 V) for an hour and visualized by UV illumination.

Cell culture

HepG2 (human hepatocellular carcinoma cell line) as caveolar endocytosis negative cell line and Hela (human epithelial cervical cancer cell line) were obtained from Pasteur Institute, Iran. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS (fetal bovine serum) and penicillin/streptomycin (50 $IU.ml^{-1}$, 500 $\mu g.ml^{-1}$) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were sub-cultured regularly using trypsin/EDTA.

In vitro transfection

The HepG2 and Hela cells were plated at 10,000 cells per well and 300 µl of media in 96 well tissue plates (Corning, USA). After incubation for 24 h, the medium was discarded, and the cells were washed once with PBS at pH 6. Miceplexes were added to either the serum free medium or 10% serum medium (pH = 6), spinned vigorously for 30 s then prepared medium was added to cells and incubated for a period of 4 h. The final concentration of pDNA was 1.5 $\mu g.cm^{-1}$. After this process, the miceplex medium was discarded and fresh culture medium (pH = 7.4), supplemented with serum and antibiotics, was added to the cells. The luciferase assay was carried out according to the manufacture's instruction (Promega, USA). Relative luciferase units (RLUs) due to luciferase activity were measured with plate reader chemiluminometer (Synergy H1, Biotech USA). RLUs normalized to protein concentration measured by bicinconinic acid (BCA) method.

Statistics

Statistical analysis was achieved using the SPSS software package (v.17). One-way ANOVA test were used with a confidence level of $p \leq 0.05$ to assess statistically significant homogeneous sub-sets.

Results and discussions

Uptakes of micelles

Hela and HepG2 cells were incubated with either 100 $\mu g.ml^{-1}$ RA-chitosan or 10 $\mu g.ml^{-1}$ free ATRA for either 1.5 or 5 h at 37 °C. To remove surface bound micelles, cells were washed with PBS several times. ATRA as positive control was easily up-taken by both of Hela and HepG2 cells after 1.5 h (Fig. 1.A-1 & A-2), While only Hela cells had up-taken RA-chitosan micelles, and no uptake was observed for HepG2 cells in that time (Fig 1.B-1). After 5 h, micelles were up-taken by both of the cells but intensity of Hela cells was higher than that of the other (Fig 1.C-1 & C-2). Results indicated that Hela cells had more potential for uptake of micelles, and uptake rate in Hela is faster than in HepG2 cells. Our results are in agreement with other studies [15, 16].

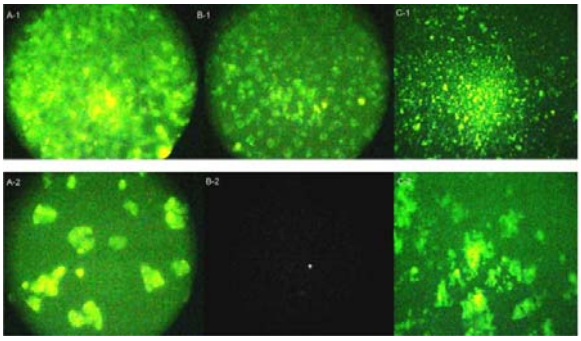

Fig. 1. uptake of free retinoic acid evaluated by fluorescent microscopy after 1.5 hour by Hela cells (A-1) and HepG2 cells (A-2), uptake of RA-chitosan micelles after 1.5 hour by Hela cells (B-1) and HepG2 cells (B-2), uptake of RA-chitosan micelles after 5 hour by Hela cells (C-1) and HepG2 cells (C-2).

Miceplexes formation

The ability of RA-chitosan micelles to make complex with DNA was assessed by agarose gel retardation assay. The electrostatic interaction between micelle and plasmid DNA (pDNA), neutralizes the negative charge of phosphate groups on DNA backbone, thus retarding the DNA mobility under the influence of electric field. The RA-micelles were incubated with pDNA at different charge ratio (CR), keeping the amount of DNA constant. The samples were analyzed on

0.8% agarose gel and charge ratio required for the formation of an electroneutral complex was investigated. As shown in Fig. 2, increasing amounts of micelles in complex leads to decreased electrophoretic mobility. The chitosan polyplex completely inhibited the migration of pDNA at CR 3 (Fig. 2A) while miceplexes showed complete retardation at CR 1 (Fig2B) which indicates stronger complex of micelle with pDNA than chitosan. This stronger complex can be explained by new Wan der Waals interaction between hydrophobic moieties of RA-chitosan and pDNA[17].

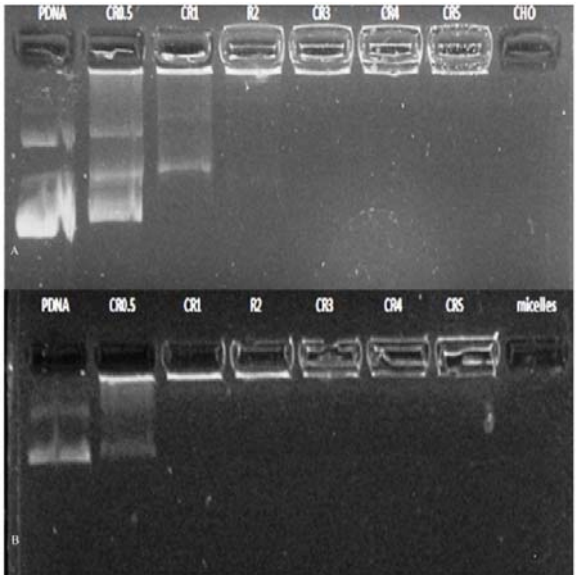

Fig. 2. Agarose gel electrophoresis of chitosan polyplex (A) and RA-chitosan miceplexes (B) at different CRs.

In vitro cell transfection studies

The various properties of RA-chitosan micelles such as small size (142.14 ± 5.06 nm), positive zeta potential (27.25 ± 6.31 mV) and capacity of loading pDNA in low CR (1) indicated that these systems possess most of the appropriate properties required to be a good transfecting agent. The transfection efficiency of themiceplexes was assessed on Hela and HepG2 cell lines, using a plasmid DNA containing reporter gene encoding luciferase. CRs greater than CR at which complete DNA retardation was observed, were used for transfection. The cells were exposed to miceplexes either in serum free medium or in medium containing 10% serum for 4 h. All transfection studies were carried out at pH, 6 as optimum pH [8]. Transfection efficiency of miceplexes at different CRs was assessed (Fig. 3 & 4). Naked DNA was considered as negative control. For both HepG2 and Hela cells, CR7 has shown higher transfection with 7.25×10^5 and 1.47×10^5 RLU.mg^{-1}, respectively (Fig. 3). With increasing CR from 3 to 7, transfection efficiency was increased, which can be attributed to complete condensation and increasing of positive charge of particles. Thereafter, transfection was dropped because of strong interaction between pDNA and micelles, and probably slow release of DNA.

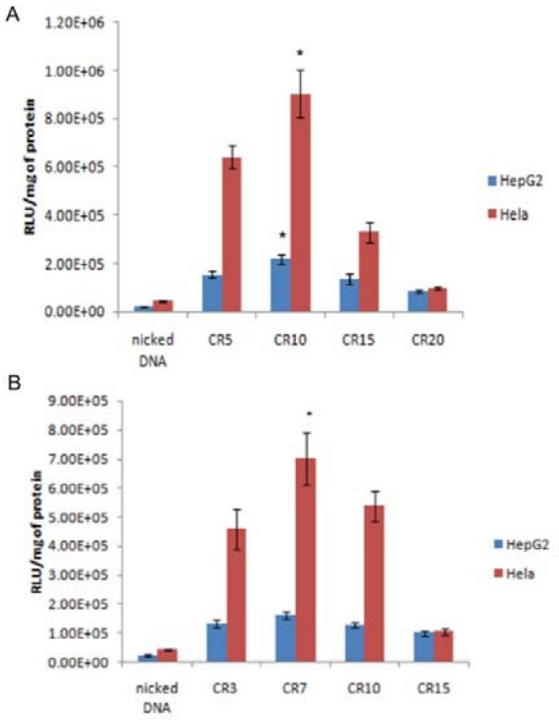

Fig. 3. Evaluation of transfection efficiency of chitosan polyplexes (A) and R-chitosan miceplexes (B) in HepG2 (blue columns) and Hela (red columns) cells. Transfection was induced for 4 h with 1.5 μg.cm^{-1} in antibiotic and serum free medium. Gene transfection was assayed after 48 h and reported as relative luciferase unit per mg of protein (RLU/mg of protein). Naked DNA was used as negative control. Significance was calculated by ANOVA (*$p \leq 0.05$).

Miceplexes showed lower transfection efficiency in both Hela and HepG2 cells grown in the serum free medium than chitosan polyplexes, with 7.25×10^5RLU.mg^{-1}versus 9.05×10^5 RLU.mg^{-1} and 1.47×10^5 RLU.mg^{-1} versus 2.16×10^5 RLU.mg^{-1}, respectively (Fig. 4). This phenomenon can be explained by stronger complex of miceplexes by the hydrophobic interactions which inhibit or

decrease release of pDNA from complex. However, it should be taken into account that hydrophobic moieties can enhance uptake of the complex and adjust sustained release of pDNA as result [17].

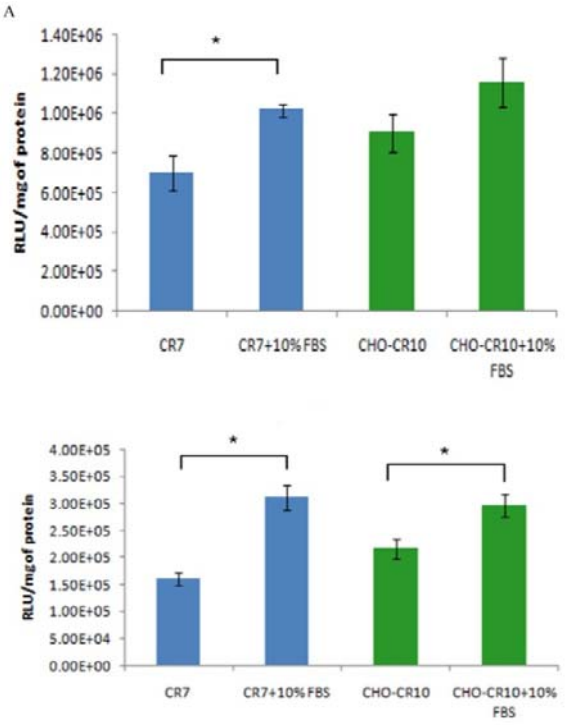

Fig. 4. Evaluation of transfection efficiency of miceplexes at CR7 and chitosan polyplexes at CR10 (CHO-CR10) in Hela (A) and HepG2 (B) cells. Transfection was induced for 4 h with 1.5 $\mu g.cm^{-1}$ in antibiotic free medium with or without serum. Gene transfection was assayed after 48 h and reported as relative luciferase unit per mg of protein (RLU.mg-1 of protein). Naked DNA was used as negative control. Significance was calculated by ANOVA ($^*p \leq 0.05$).

Transfection of both miceplexes and polyplex in HepG2 was significantly lower than Hela cells in the same condition (Fig. 3), which is not a surprising result. Lower and slower uptake of micelles by HepG2 cells could be one of the reasons for lower transfection efficacy in this cell line. Furthermore, pinocytosis and clatrin mediated endocytosis are the main ways for uptake of micelles in HepG2, and endosomal escape in this cell is the main barrier, especially for polymers with low proton-sponging capacity (e.g chitosan) [8].

Stability and transfection of the non-viral vector in the presence of serum are other challenges in non-viral gene delivery. Chitosan mediated gene delivery systems are the rare systems which can increase transfection in the presence of serum [18]. To obtain this ability in miceplexes, transfection of miceplexes at CR7 was investigated and compared with transfection of chitosan polyplexes at CR10. As it is shown in Fig.4 transfection efficacy of miceplexes and polyplexes in both of Hela (Fig.4.A) and HepG2 (Fig.4.B) was enhanced in the presence of 10% fetal bovine serum (FBS) comparing to serum free medium. In both of the cell lines, transfection of miceplexes was higher than transfection of polyplexes in 10% FBS medium. On the other hand, conjugation of hydrophobic moieties to chitosan could increase transfection in the presence of serum. Our results are in agreement with other similar studies [17, 19].

Conclusion

Here we successfully evaluated RA-chitosan micelle as a non-viral vector. Uptake study has indicated that micelles can be up-taken by Hela cells faster and more efficient than by HepG2 cells. Micelles condensed pDNA at CR lower than CR of chitosan polyplexes. Miceplexes exhibited lower transfection efficiency when compared with chitosan in Hela and HepG2 cells. Transfection in both of miceplexes and chitosan polyplexes in the presence of 10% serum was increased but miceplexes have shown higher transfection. These properties, all together make RA-chitosan a safe and effective vector for in vitro and potentially in vivo gene delivery. According to our results in previous study for delivery of hydrophobic anticancer drug by RA-chitosan micelles, they also have the potential for delivery of anticancer drugs, and can be used for co-delivery of drug and gene.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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