Functional Nanoparticles for Oral Delivery of Anti-Angiogenic Protein

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ABSTRACT SUMMARY
Chitosan (CS) and poly(γ-glutamic acid) (γ-PGA) have been chemically modified with arginine (Arg) and taurine (Tau), respectively. The synthesized CS-N-Arg and γ-PGA-Tau conjugate polymers were used to prepare self-assembled nanoparticles (NPs) which responded to changes in the pH of simulated gastrointestinal (GI) tract media. Evaluation of test NPs in enhancing the intestinal paracellular transport of AE-941 protein, a naturally occurring inhibitor of angiogenesis, was investigated in vitro in Caco-2 cell monolayers. The paracellular transport of AE-941 protein was improved by using the NPs as protein delivery carriers, whereas the transported AE-941 protein was effective in competing with VEGF for binding to the human umbilical endothelial cells (HUVEC) surface and subsequently inhibited HUVEC tube formation.

Keywords: chitosan, paracellular transport, pH-responsive, NPs

INTRODUCTION
AE-941 protein is a water-soluble extract derived from shark cartilage with evidence of antiangiogenic and antitumor activity.¹,² The angiogenic protein selectively inhibits matrix metalloproteinases (MMPs) and prevents the binding of VEGF to its receptor, results in significantly inhibiting angiogenesis. Unfortunately, the poor oral availability of AE-941 protein results in failure of effective cancer therapies. Hence, vehicles that can protect the loaded protein from destruction in the gastrointestinal (GI) tract and enhance their intestinal absorption are desperately needed.

This study attempts to integrate the improved pH-responsive property and TJ opening activity in the CS-N-Arg/γ-PGA-Tau NPs for oral antiangiogenic protein delivery. The pH-responsive properties of the self-assembled NPs and their protein release properties were examined over a wide pH range (pH 1.2–7.4, simulating the pH in the gastrointestinal tract). Effects of chemical modification of CS and γ-PGA on TJ opening and transport enhancement of AE-941 protein were evaluated in vitro in Caco-2 cell monolayers. The transported AE-941 protein showed an ability to compete against the binding of VEGF to its receptor in human umbilical endothelial cells (HUVEC) and the effect of inhibition of HUVEC tube formation was studied.

EXPERIMENTAL METHODS
Preparation of Protein-Containing NPs
CS-N-Arg and γ-PGA-Tau conjugate polymers were respectively synthesized according to the literatures. A sample of 1 mg of AE-941 protein was dissolved in 1 ml of DI water to make a 1 mg/ml of stock solution of AE-941 protein. The AE-941 protein stock solution (2 mg/ml, 1 ml) was premixed with an aqueous γ-PGA-Tau (2 mg/ml, 1 ml). Subsequently, the mixed solution was added into an aqueous CS-N-Arg (1.2 mg/ml, 10 ml) under magnetic stirring at room temperature. To determine their AE-941 protein loading content and loading efficiency, NPs were collected by ultracentrifugation at 15000 rpm, 4°C for 50 min and the AE-941 protein concentration in the supernatant was assayed by Brandford protein assay kit (Bio-Rad Protein Dye Reagent; Bio-Rad).

Matrigel tube formation assay
The 12-well receiver plates were precoated with 500μl/well Matrigel (10 mg/mL) (BD Bioscience, USA). The gel was allowed to solidify for 30 min at 37°C. The treatment of Caco-2 cells with AE-941 protein-loaded NPs (5-25 μg AE-941 protein equivalent/mL) started at 24h after seeding HUVEC. Prior to the...
transport experiment, the insert Caco-2 cells were washed twice with Hank’s balanced salt solution (HBSS) and pre-equilibrated for 30 min with HBSS buffered at pH 7.4. After removing the HBSS, the inserts transfer to the 12-well receiver plates. The receiver plates contained HUVEC (2×10^5 cells/well) which were seeded on the Matrigel and cultured in M199 medium supplemented with 10% FBS and 30μg/mL endothelial cell growth supplement (ECGS). At the same time, insert Caco-2 cells were treated with new HBSS (pH 7.0) containing AE-941 protein-loaded NPs or blank NPs in the apical compartment for 2 h. After removing the NPs in the apical compartment, the HUVEC cells were further cultured in the receiver plates for 48 h. The inhibition rate was calculated using the following formula: Inhibition rate of tube formation = [1-(tube length sample/tube length control)] ×100%.

RESULTS AND DISCUSSION

The turbidity change took place sharply when γ-PGA-Tau was added into the CS-N-Arg aqueous solution. This result suggests that NPs were self-assembled upon the addition of an aqueous γ-PGA-Tau into an aqueous CS-N-Arg under magnetic stirring at room temperature. As shown in Table 1, the diameters of the prepared NPs were in the range of 107–147 nm with a negatively or positively charged zeta potential, depending on the relative concentrations of CS-N-Arg and γ-PGA-Tau used (Table 1).

Table 1. Mean particle sizes, zeta potential values and polydispersity indices of CS/γ-PGA and CS-N-Arg/γ-PGA-Tau NPs (n = 5 batches).

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<thead>
<tr>
<th></th>
<th>Size (nm)</th>
<th>Zeta Potential (mV)</th>
<th>PI*</th>
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<tbody>
<tr>
<td>CS/γ-PGA = 5:1</td>
<td>104.1±1.2</td>
<td>34.2±2.5</td>
<td>0.11±0.02</td>
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<tr>
<td>CS-N-Arg/γ-PGA-Tau</td>
<td></td>
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<tr>
<td>5:1</td>
<td>147.3±4.2</td>
<td>36.9±2.8</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>1:1</td>
<td>106.6±5.4</td>
<td>20.4±3.5</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td>1:5</td>
<td>118.9±3.7</td>
<td>-11.9±4.8</td>
<td>0.12±0.03</td>
</tr>
</tbody>
</table>

* Polydispersity Index

AE-941 proteins transported across Caco-2 cell monolayers by NPs (loaded with 5, 10 and 25 μg AE-941 protein eq/mL, in the apical compartment), demonstrated 6.5%, 23.6% and 95.4% inhibition rate of tube formation (Figure 1), which is less effective in the inhibition of HUVECs tube formation as compared to those of controls (HUVECs directly incubated with AE-941 proteins) (P < 0.05). This was attributed to the fact that the extent of inhibition depends not on the amount of AE-941 proteins added to the apical side of the cell monolayer but on the cumulative amount transported for AE-941 proteins across Caco-2 cell monolayers.

Figure 1. The total tube length of HUVECs measured on Matrigel, in presence of 5, 10 and 25 μg AE-941 protein eq/mL of NPs (n = 5 batches)

CONCLUSION

A pH-responsive NP system self-assembled by CS-N-Arg and γ-PGA-Tau could be used for oral delivery of AE-941 protein was successfully prepared in the study. The transported AE-941 protein was effective on competing with VEGF for binding to its receptors on HUVECs and subsequently inhibited the HUVECs tube formation.

REFERENCES


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