Hyaluronic Acid Nanogel as a Protein Delivery System

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PURPOSE

Hyaluronic acid (HA) nanogel has hyaluronic acid in the main chain, a hydrophilic, biocompatible material with low toxicity, and a hydrophobic group in the side chain. A nano-ordered gel is spontaneously formed, due to its hydrophilic-hydrophobic interactions, in which it is possible to load small molecules, peptides, and proteins.(1,2) Unique characteristics are predicted, such as improved targeting ability, reduced toxicity and solubility of the active pharmaceutical ingredients (APIs). HA nanogel can load various proteins; however, the correlation between the physicochemical property of proteins and the loading capacity has not been investigated. In order to explore the function of HA nanogel-protein complex preparations, we investigated stability of protein activity, and inhibition of aggregation and denaturation with Cholesteryl-modified Hyaluronic Acid (CHHA) as our HA nanogel.

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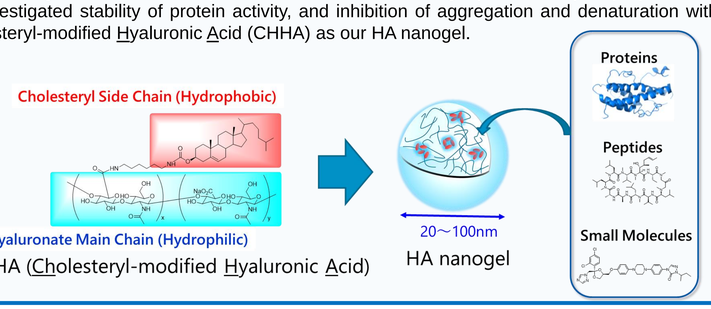


OBJECTIVES

Aiming to understand the interaction between the protein and HA nanogel, the relationship between TSA analysis and the loading capacities, and the influence on the aggregation and activity of the proteins when the nanogels were encapsulated were examined. This study contributes to the development of formulations with high protein stability.

METHODS

For this study, we used CHHA whose degree of substitution by the cholesteryl group is 12 % in HA units, and whose molecular weight is 80 kDa.

Sustained release of hGH from HA nanogel/hGH complex in VIVO

CHHA solution and hGH solution in 10wt% sucrose was mixed in various concentration and it was incubated at 37C for 24 hours. The CHHA/hGH formulations were injected subcutaneously into the back of seven-week-old male Sprague-Dawley with a 25-gauge needle at a dose in the table of figure 1. Blood was collected from the jugular vein and rhGH levels in plasma were determined using an hGH enzyme-linked immunosorbent assay (ELISA) kit.

TSA (Thermal Shift Assay) Analysis

SYPRO® Orange protein gel stain (50 times diluted, 1 µL) was added to the protein solution (0.151 mg/mL, 19µL). The fluorescence intensity was measured by the real-time polymerase chain reaction (RT-PCR), with a scan mode of FRET.

Loading Proteins in HA Nanogels

Protein was incubated with CHHA at 37 ºC for 24 hours ([protein] = [CHHA] = 1 mg/mL), and then the solution (200 µL) was put into ×5 PBS buffer (50 µL) to precipitate the complex of CHHA and protein. After centrifuging at 9000*g* for 10 min, the protein in the supernatant was subjected to GPC measurement to determine the amount of encapsulated protein. Loading capacity of protein = [amount of encapsulated protein] / [amount of CHHA].

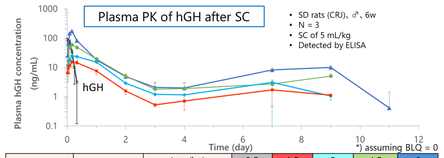
Antibody Stability in HA nanogels

Antibody (anti-OVA antibody, IgG from rabbit) was incubated with or without CHHA, at 4 or 80 ºC for 24 hours ([antibody] = 4 µg/mL, [HA nanogel] = 6 mg/mL), and O.D. was measured using a plate reader, to evaluate the thermal stability of antibodies in HA nanogel.

RESULTS

Sustained release of hGH from HA nanogel/hGH complex

- HA nanogel is precipitated under physiological salt condition (ex. PBS) by salt-induced association.
- **Releasing of hGH encapsulated in HA nanogel extended for 10 days**, and hGH concentration in plasma is depended on dose.



Formulation	hGH	(mg/kg)	0.5	1.5	3	4.5	6
		(Loading % w/w)	-	5	10	15	20
		(mg/mL)	0.1	0.3	0.6	0.9	1.2
	HA nanogel	(mg/mL)	-	6	6	6	6
PK parameters	Cmax	(ng/mL)	99.1	16.8	27.8	58.8	173.2
	AUCinf	(ng hr/mL)*)	332	562	970	1711	2963
	MRTinf	(hr)	2.6	58.4	55.7	53.1	66.3
	BA (*vs* SC)	(%)	–	58.2	50.3	59.1	76.8

Figure 1. Plasma pharmacokinetics (PK) data of hGH with/without HA nanogel after subcutaneous injection.

Speculated mechanism of sustained release from HA nanogels

- HA nanogel/hGH was precipitated in PBS or PBS with bovine serum albumin (BSA) and then the concentration of hGH in supernatant was determined by GPC after 1.5, 6, and 24 hours.
- We could not observe any hGH in PBS supernatant, but that in PBS/BSA was a gradually increasing over time.
- We estimate **that hGH in HA nanogel was released by replacement with internal proteins**.

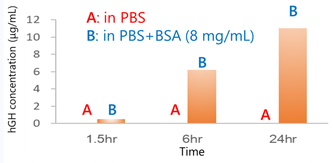


Figure 2A. The hGH concentration in supernatant in PBS or PBS/BSA solution

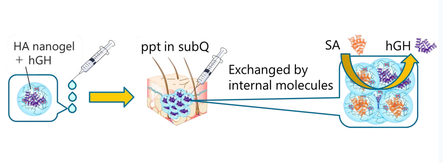


Figure 2B. The estimation mechanism of releasing hGH form HA nanogel.

TSA Analysis of Proteins and Loading Capacities in HA nanogel

- The denaturation temperatures and fluorescence intensities at 37 ºC (encapsulation temperature) in TSA measurement are compared with loading capacities.
- The molecular weight of protein did not correlate with the loading capacity.
- **Proteins with a lower denaturation temperature tend to be more entrapped in HA nanogel.**
- Especially proteins such as conalbumin and aldolase, which **have higher fluorescence intensity at 37 ºC, showed a higher loading capacity**. (Higher fluorescence intensity means

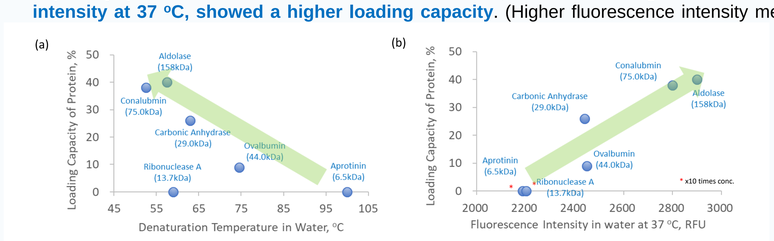


Figure 3. The relationship between loading capacity of protein in HA nanogel and (a) denaturation temperature or (b) fluorescence intensity in water at 37 ºC.

Anti-Aggregation of Proteins in HA Nanogel

- Conalbumin solution was stored at 58 ºC (denaturation temperature) with/without HA nanogel.
- After 12 hr of incubation, aggregation was observed in conalbumin-only solution, **while the HA nanogel containing conalbumin was transparent.**
- This result indicated that HA nanogel can make a complex with proteins, which prevents protein molecules from closely interacting.

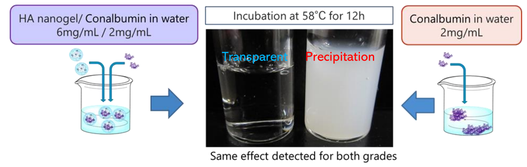


Figure 4. The solution of conalbumin with (left) and without (right) HA nanogel after incubating at 58 ºC for 12 hours.

Activity Retention after Releasing from HA Nanogel

- HA nanogel can load 20 % hGH by weight.
- After releasing hGH from HA nanogel/hGH complex by HP-β-CD, **its hGH activity was equal or slightly higher than that of non-gel-loaded hGH**.
- HA nanogel might work as artificial chaperone, like cholesterol pullulan (CHP) nanogel.

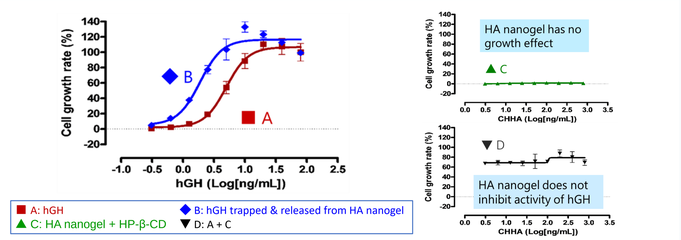


Figure 5. Cell growth rate of various hGH concentration with or without HA nanogel.

Protecting Antibodies from Denaturation

- The titer of IgG antibody dramatically decreased after treatment at 80 ºC. However, **the solution with HA nanogel showed a much higher titer**.
- The comparison of titer at 0.2 of O.D. between the solution with and without HA nanogel, IgG with HA nanogel shows 8.1 times higher titer than that without HA nanogel.

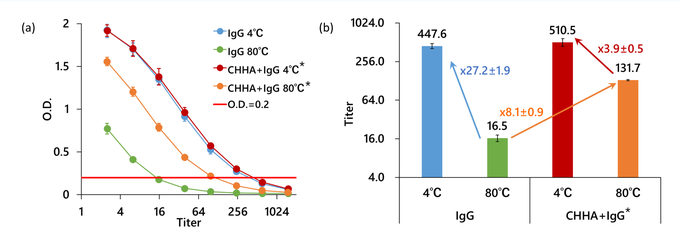


Figure 6. (a) The profile between titer and O.D. at 450 nm of IgG or IgG/CHHA after incubating at 4 or 80 ºC. (b) Comparison of the titer at O.D. of 0.2. *IgG was released from HA nanogel/IgG complex by HP-β-CD

CONCLUSIONS

1. **Sustained release for 10 days was observed for the formulation with HA nanogel.**
2. **Proteins with higher hydrophobicity are easier to entrap, in HA nanogel. In addition, utilizing TSA measurement, we estimated the loading ability of proteins and the best conditions of formation for the HA-nanogel/protein complex.**
3. **The activity of hGH seemed to increase after loading in HA nanogel. Also, HA nanogel made protein more stable under high temperature condition and avoided aggregation.**
4. **HA nanogel can protect antibodies under high temperature conditions; encapsulation in HA nanogel might help to extend the storage period of antibody solutions.**

REFERENCES

1) Macromol. Biosci. 2012, 12, 475 – 483
2) WO2010-053140

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