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A decrease in moisture absorption–retention capacity of *N*-deacetylation of hyaluronic acid

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Abstract The linear non-sulfated glycosaminoglycan, hyaluronic acid (HA), is widely distributed throughout connective, epithelial and neural tissues etc., and is of great importance in tissue hydration, lubrication and cellular function. Along with the age growth, HA will lose its acetyl groups under action of HA *N*-deacetylase *in vivo*. However, the biological consequence of this physiological process remains largely unknown. Herein two highly *N*-deacetylated HAs, dHA-6 and dHA-10 were generated *via* the NH_2NH_2-HIO_3 procedure. Their molecular weights were estimated to be 24 and 16 kDa by high performance gel-permeation chromatography (HPGPC), and the *N*-deacetylation degrees were 79.4 % and 93 % respectively, as determined by ^1H nuclear magnetic resonance (NMR). The study on moisture-absorption (*Ra*) and -retention (*Rh*) abilities demonstrated that the *Ra* values of dHAs under conditions of 81 % or 43 % relative humidity, as well as the *Rh* values of dHAs under dry condition or 43 % relative humidity, were significantly smaller than that of their respective re-*N*-acetylated products. The decline of moisture-absorption and –retention capacity after HA *N*-deacetylation were consistent with the appearance of unsolvated amides remained in the *N*-deacetylated products, as indicated by circular dichroism (CD) spectroscopy. Our findings implied that HA *N*-deacetylation, in addition to the decrease of HA contents in the elderly persons, might account for manifestations of naturally aged skin, such as laxity, sagging, and wrinkling.

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Abbreviations

HA	hyaluronic acid
CD	circular dichroism
RH	relative humidity
Ra	moisture-absorption abilities
Rh	moisture-retention abilities
HPGPC	high performance gel-permeation chromatography
NMR	nuclear magnetic resonance
dHA-6	HA *N*-deacetylated product resulted from 6 h of hydrazinolysis
dHA-10	HA *N*-deacetylated product resulted from 10 h of hydrazinolysis
HA-6	re-*N*-acetylated product of dHA-6
HA-10	re-*N*-acetylated product of dHA-10

Introduction

Hyaluronic acid (HA), also called hyaluronan or hyaluronate is a nonsulfated glycosaminoglycan (GAG) which consisted of alternating residues of β-4 linked D-glucuronic acid and β-3 linked *N*-acetyl-D-glucosamine [1, 2]. In mammals, HA is distributed widely throughout connective, epithelial, and neural tissues and is abundant in heart valves, skin, skeletal tissues, the vitreous of the eye, the umbilical cord, and synovial fluid [3, 4]. As an component of intercellular matrix, HA carries out numerous functions that are essential for survival, differentiation, proliferation, motility and intercellular communication of various cell types, and is

of crucial importance for the development and architecture of tissues and organs [5].

In the skin, HA plays an important role in 1) holding moisture [6], 2) increasing viscosity and reducing permeability of extracellular fluid, 3) contributing to mechanical resilience and suppleness of the skin, 4) regulation of tissues repair [7], 5) regulation of movement and proliferation of cells, and 6) regulation of immune and inflammatory responses [8–10]. Interestingly, age can cause substantial changes in the content and structure of the skin HA [11–13]. On one hand, after peaking in adolescence or early adulthood, skin HA content declines with age. On the other hand, HA loses its *N*-acetyl moieties as a function of age [12, 13], which is almost completely absent by the middle of the seventh decade of life. At the present, the biological significance of this HA *N*-deacetylation process remains largely unknown.

In this study, highly *N*-deacetylated HAs were prepared *via* the NH_2NH_2-HIO_3 procedure and further characterized by high performance gel-permeation chromatographic (HPGPC), ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The moisture absorption–retention capacities and circular dichroism (CD) spectroscopy of these *N*-deacetylated HAs were explored.

Experimental

Reagents and materials

Sodium hyaluronate (~2,000 kDa) from chicken combs, iodic acid, hydroiodic acid and hydrazine monohydrate were purchased from Aladdin Chemical Reagent Company, China. Hydrazine monohydrate is dried by refluxing with an equal weight of sodium hydroxide pellets for 2 h in a current of dry nitrogen and distilled to obtain anhydrous hydrazine. All other chemicals were of analytical grade as available.

Preparation of highly *N*-deacetylated HAs

This was performed according to the procedure developed by Höök [14] and Dahl [15] with minor modifications. As shown in Fig. 1, sodium hyaluronate was dissolved in anhydrous hydrazine containing 1 % hydrazine sulfate in a glass tube. After deoxygenation of the solution by bubbling in nitrogen, the tube was sealed and the reaction was conducted at 105 °C for 6 h or 10 h. The polymeric products were precipitated by cold ethanol and were re-dissolved in aqueous 5 % acetic acid, to which 0.5 M iodic acid (HIO_3) was added. The mixtures were kept in a bath at 4 °C for 2 h. The excess HIO_3 was removed by the addition of 57 % hydroiodic acid. The colored solutions were extracted with ethyl ester repeatedly, and the aqueous layers recovered were neutralized with 0.2 M sodium hydroxide. The *N*-deacetylated HAs were obtained after ethanol precipitation, thoroughly dialysis and freeze-dried. The products resulted from 6 h and 10 h of hydrazinolysis were designated as dHA-6 and dHA-10 respectively.

Re-*N*-acetylation *of* dHA-6 and dHA-10 [16]

This was achieved by treating dHA-6 and dHA-10 with 5 % (v/v) acetic anhydride in the saturated sodium bicarbonate solution for 30 min at room temperature. After dialysis and freeze-dried, the fully *N*-acetylated products, designated as HA-6 and HA-10, were obtained.

Determination of molecular weight

As described previously [17], the molecular weight was determined by high performance gel permeation chromatography (HPGPC) on three columns (Waters Ultrahydrogel 250, 1,000 and 2,000; 30 cm×7.8 mm; 6 μm particles) in series. The columns were calibrated with T-series Dextrans (5.2, 10, 48.6, 668, 2,000 kDa). Sodium acetate (3 mM) was used as eluant and the flow rate was kept at 0.5 mL/min. A 100 μL aliquot was injected for each run. The calibration curve of Log (Mw) *vs*. elution time (T) is: $\mathrm{Log(Mw)} = -0.1171\mathrm{T} + 10.45$.

NMR analysis

Samples were deuterium-exchanged twice. The ^{1}H and ^{13}C NMRspectra of samples dissolved in deuterium were recorded with a Bruker AM 500 spectrometer with a dual probe in the FT mode at room temperature.

Moisture absorption test [18, 19]

Each sample was freeze-dried for 24 h and was kept in a silica gel drier overnight. Samples were put into desiccators that contained a saturated aqueous solution of ammonium sulfate (81 % relative humidity, RH) or sodium carbonate (43 % RH) for different periods at room temperature. The moisture absorption ability (*Ra*)

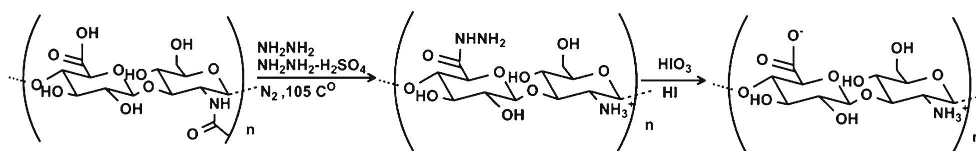

Fig. 1 Scheme for preparation of highly *N*-deacetylated HAs

was evaluated by the percentage of increase in weight of dry samples:

$$Ra(\%) = 100 \times (Wn - Wo)/Wo$$

W_0 and W_n were the weights of samples before and after putting it into the desiccators, respectively. The *Ra* values of each sample in triplicate were averaged.

Moisture retention test [20, 21]

As above, each freeze-dried sample was put into a silica gel drier overnight. 10 % water was added to each sample. Samples were then put into desiccators that contained a saturated aqueous solution of sodium carbonate or silica gel for different periods at room temperature. The moisture retention ability (*Rh*) was evaluated by the percentage of residual water of wet sample:

$$Rh(\%) = 100 \times (Hn/Ho)$$

H_0 and H_n were the weights of water in the samples before and after putting it into the desiccators, respectively. The *Rh* values of each sample in triplicate were averaged.

Circular dichroism (CD) spectroscopy [22]

Measurements were carried out by using a CD6 dichrograph (Jobin Yvon Optics and Spectroscopy, France) coupled to a microcomputer. Samples at the concentration of 1.0 mg/mL were introduced into the cell with optical pathway of 1 or 2 mm, and thermostated at 25 °C within 0.1 °C. The wavelength was varied in the 170–250 nm spectral range with an interference band of 2 nm and the integration time was fixed at 0.2 s. The scan was repeated three times for each sample and the recorded spectra were averaged.

Results

Preparation and characterization of highly *N*-deacetylated HAs

The HA *N*-deacetylated products (dHAs), dHA-6 or dHA-10 was obtained after 6 h or 10 h hydrazinolysis of HA, respectively, (see Experimental section for details). Both dHA-6 and dHA-10 were eluted as a symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC) (Fig. 2). Their molecular weights were estimated to be 24 and 16 kDa, in reference to standard dextrans. Interestingly, the molecular weights of re-*N*-acetylated products (HAs), HA-6 and HA-10, were 34 and 27 kDa, respectively. The dramatic disparity of polydispersity between HAs and their respective dHAs, as shown on HPGPC profiles implied that *N*-acetyl groups might be essential in orchestrating the fine space structure of naturally existed HA.

The *N*-deacetylation degree was estimated by the ^{1}H-NMR spectra of HAs and dHAs. As shown in Fig.3A, in one repeating unit of native HA polymer there are 3 methyl protons (acetyl group) every 2 anomeric protons, which means the integral ratio between the signals at 1.8–2.0 ppm and that at 4.3–4.7 ppm is 3:2 theoretically. Expectedly, HA-6 and HA-10 were fully *N*-acetylated as indicated by molar ratio of methyl and anomeric protons in ^{1}H-NMR spectra of dHA-6 and dHA-10 (Fig. 3). As calculated, the *N*-deacetylation degrees of dHA-6 and dHA-10 were 79.4 % and 93 %, respectively. The ^{13}C-NMR spectra (Fig.3B) further demonstrated some acetyl groups remained in dHA-6, instead of dHA-10.

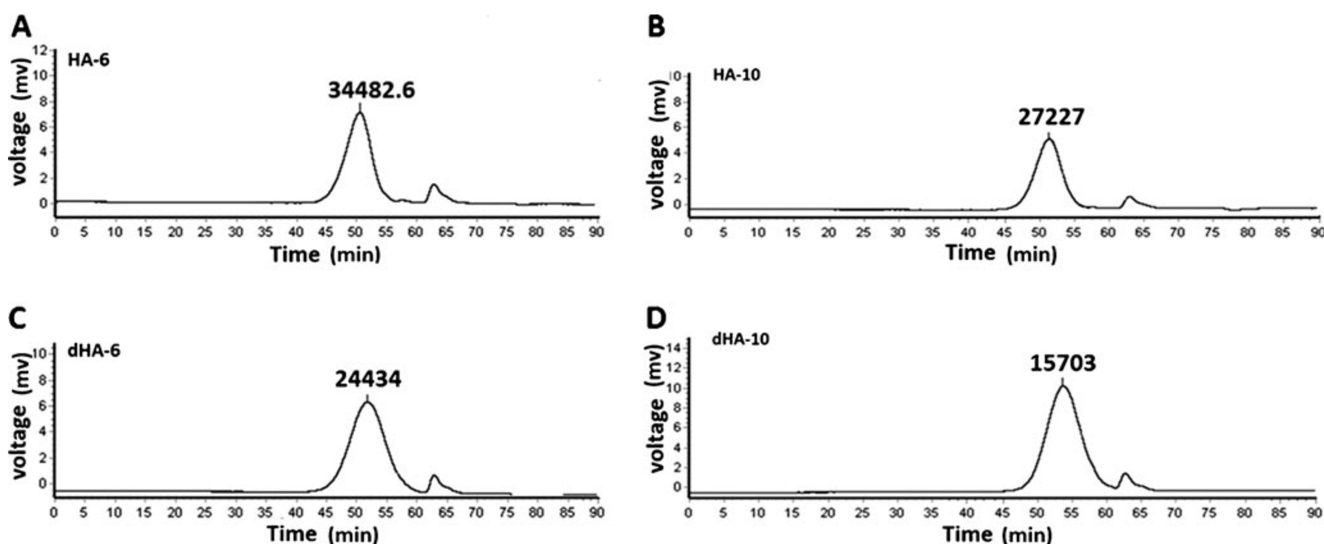

Fig. 2 HPGPC chromatogram on ultrahydrogel columns of HA-6 (**a**), HA-10 (**b**), dHA-6 (**c**) and dHA-10 (**d**). The numbers that are listed on top of the peaks represent the molecular weights, calculated from the calibration curve of Log(Mw) *vs*. elution time by using standard dextrans with known molecular weights

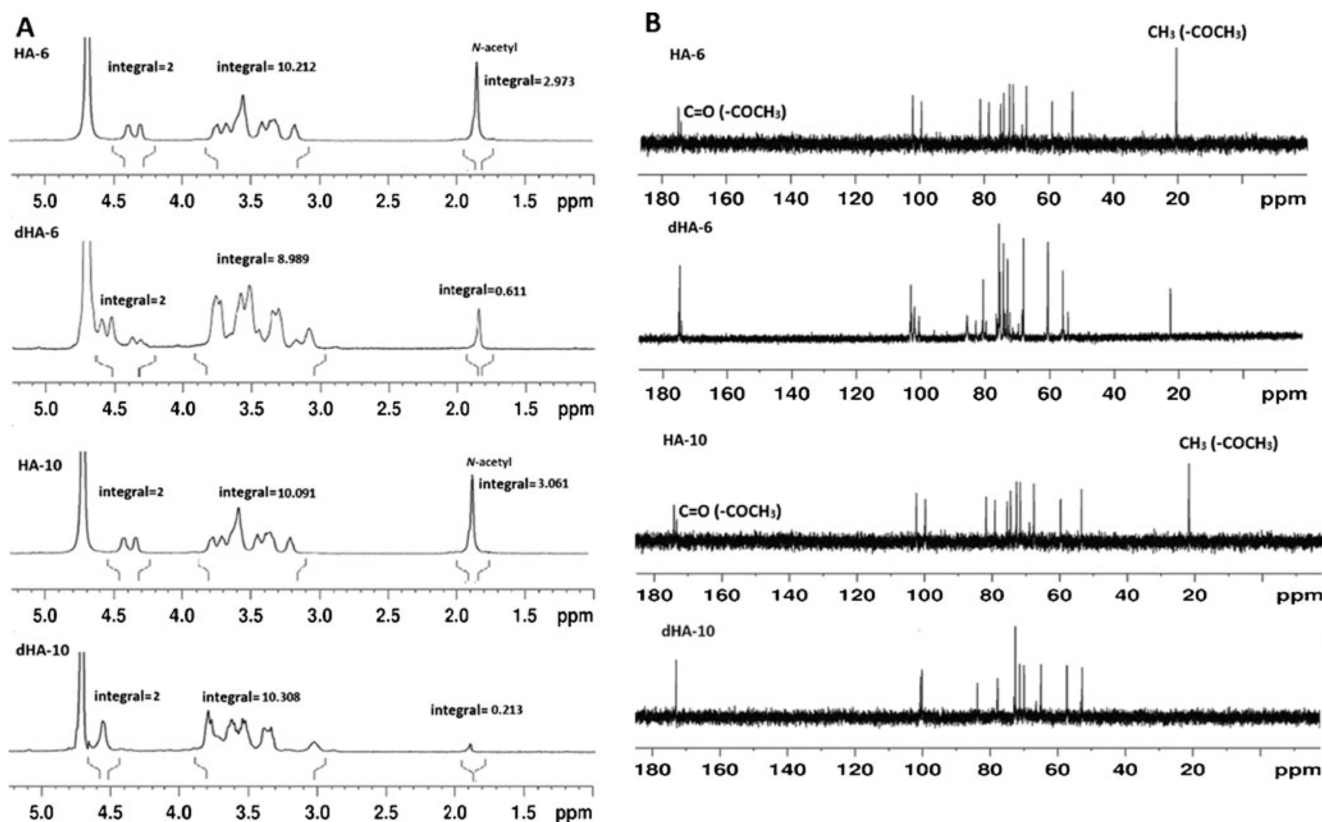

Fig. 3 ^{1}H(A) and ^{13}C(B) NMR spectra of HA-6,dHA-6, HA-10 and dHA-10. The ^{1}H and ^{13}C NMR (500 MHz) spectra were recorded in the FT mode at room temperature

Comparison of moisture-absorption (*Ra*) and moisture-retention (*Rh*) abilities of dHAs and its respective HAs

The moisture-absorption properties of original HA (~2,000 kDa), dHA and HAs were shown in Fig. 4. At both 81 % and 43%RH, for all samples tested, the weight of moisture absorbed increased rapidly at the first stage, slowed down in the latter stage, and then became constant. Apparently, at both 81 % and 43 % RH, original HA demonstrated much stronger moisture-absorption properties than HA-6 and HA-10. At 81 % RH, the maximum *Ra* values of dHA-6 and dHA-10 were 25.63 % and 23.43 %, while that of HA-6 and HA-10

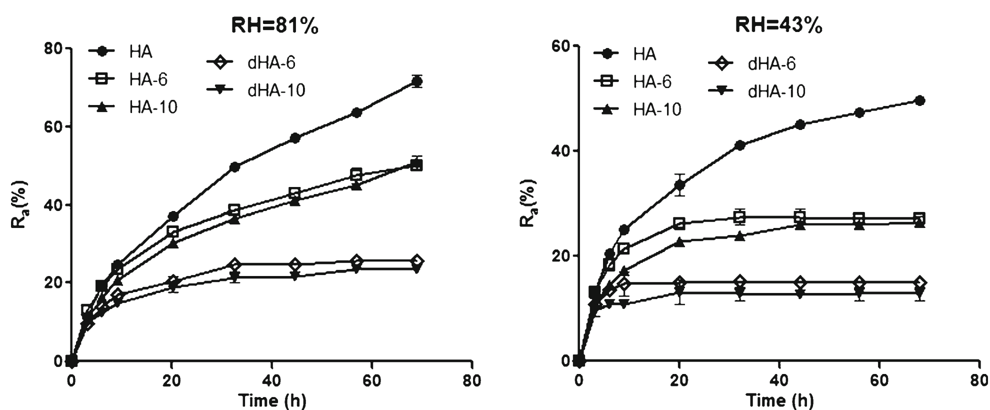

Fig. 4 Moisture absorption abilities (*Ra*) of original HA, HAs and dHAs. Samples were put into desiccators that contained a saturated aqueous solution of ammonium sulfate (81 % RH) or sodium carbonate (43 % RH) for different periods at room temperature. The moisture absorption ability (*Ra*) was evaluated by the percentage of increase in weight of dry samples. The *Ra* values of each sample in triplicate were averaged. A representative of three experiments was shown

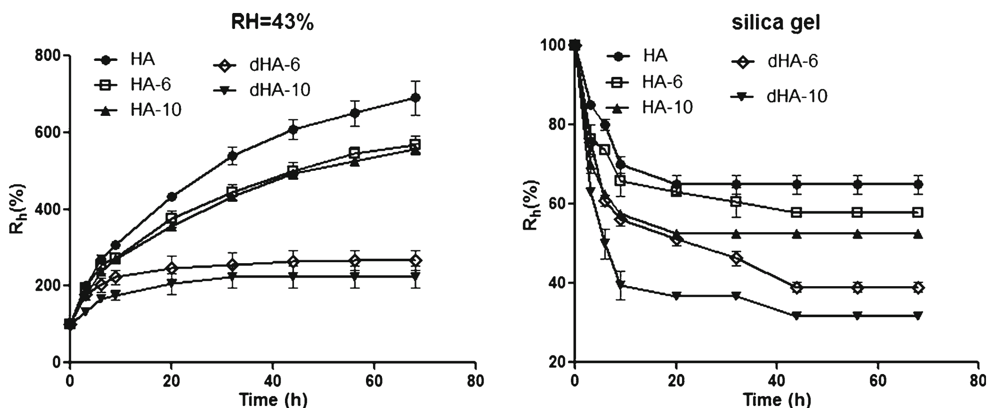

Fig. 5 Moisture retention abilities (*Rh*) of original HA, HAs and dHAs. Samples were put into desiccators that contained a saturated aqueous solution of sodium carbonate (43 % RH) or silica gel for different periods at room temperature. The moisture retention ability (*Rh*) was evaluated by the percentage of residual water of wet samples. The *Rh* values of each sample in triplicate were averaged. A representative of three experiments was shown

were 50.12 % and 50.875 %, respectively. At 43 % RH, the maximum *Ra* values of dHA-6 and dHA-10 were 15.07 % and 12.85 %, in comparison to 27.24 % and 26.58 % of HA-6 and HA-10 respectively.

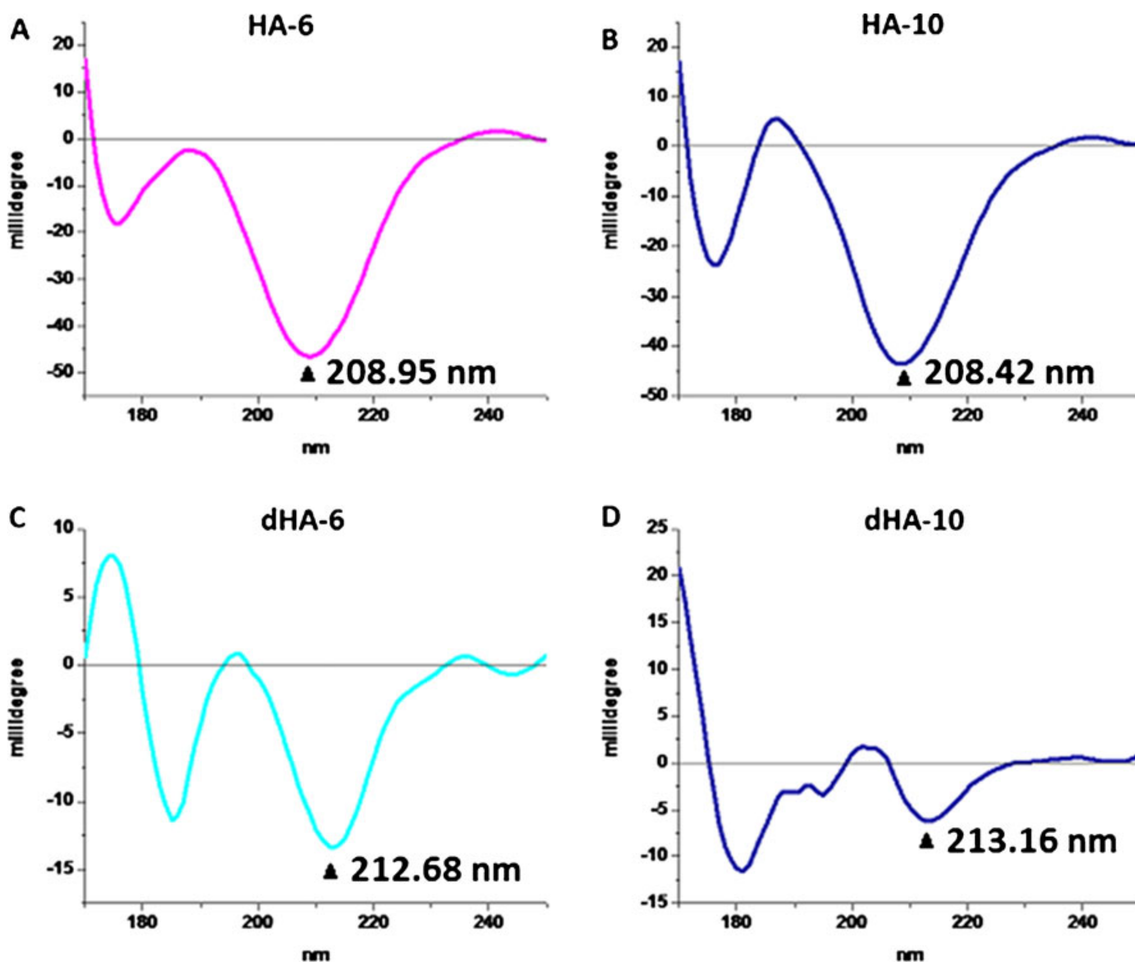

Fig. 6 CD spectra of HA-6 (A), HA-10 (B), dHA-6 (C) and dHA-10 (D) at 1 mg/mL at 25 °C. Measurements were carried out by using a CD6 dichrograph coupled to a microcomputer, and each sample was scanned three times and the recorded spectra were averaged. Measurements were carried out by using a CD6 dichrograph (Jobin Yvon Optics and Spectroscopy, France) coupled to a microcomputer

In the results of the moisture-retention test (Fig. 5), the weights of residual moisture in the wet samples increased with time when the relative humidity was 43 %. In the silica-gel desiccators, the residual moisture in all samples decreased quickly and became constant after 20 h. It was obvious that the dHA-6 and dHA-10 demonstrated much lower moisture-retention abilities than their respective re-*N*-acetylated products (HA-6 and HA-10). Taken together, our results indicated that *N*-acetyl groups played an essential role in the moisture absorption and moisture-retention abilities of HA, the most powerful natural moisturing ingredient known to science.

CD spectroscopy analysis

In the CD Spectra (Fig. 6), both HA-6 and HA-10 at 1 mg/mL exhibited a negative CD band centered near 210 nm (< 210 nm), which was attributed primarily to the n–π* transition of the amide chromophore of the acetamido group, with a smaller contribution from the n–π* transition of the carboxylate chromophore. This observation was consistent with that of aqueous native HA with larger molecular size [23]. After *N*-deacetylation, the intensity of the negative CD band indicative of n–π* transition of the amide group significantly decreased in the CD spectra of dHA-6 and dHA-10 (Fig. 6).

Different from HA-6 and HA-10, both dHA-6 and dHA-10 demonstrated a negative CD band centered above 210 nm (Fig. 6). This shift resulted from unsolvated amides (amides with no attraction and association of water molecules), which typically had CD bands at higher wavelengths [24]. The appearance of 'unsolvated' amides in dHA-6 and dHA-10 reflected a decrease in moisture absorptio-retention capacity after HA *N*-deacetylation at the molecular level.

Discussion

Hyaluronic acid is an important constituent of the extracellular matrix, which seems to be a functionally very diverse system [25]. Skin HA will lose *N*-acetyl groups under the action of HA *N*-deacetylase as a function of age [11], and will generate completely *N*-deacetylated products by the middle of the seventh decade of life [11–13]. However, to our knowledge, the biological significance of this HA *N*-deacetylation process still waits to be explored.

To obtain the highly *N*-deacetylated products, HA was hydrazinolyzed with the NH_2NH_2-HIO_3 procedure. The molecular weights of the end products, dHA-6 and dHA-10 were determined to be about 24 and 16 kDa by HPGPC (Fig. 2). The *N*-deacetylation degrees were 79.4 % for dHA-6 and 93 % for dHA-10, according to ^{1}H nuclear magnetic resonance (Fig. 3).

It is well-known that HA is the key molecule involved in skin moisture due to its strong water-retaining ability. Structural evidence for the HA-H_2O interaction showed that when HA goes into an aqueous solution, the intra-residue hydrogen bonds get augmented with hydrogen bonds involving the solvent molecules. Furthermore, four intra-residue hydrogen bonds are augmented with a water bridge between an acetamido group and a carboxylic group using two disaccharide units [25]. Although it has been reported that carboxylic groups were an effective group to enhance water-retention capacity [20], our findings indicated that the loss of *N*-acetyl groups in HA resulted in a significant decrease of both moisture absorption and retention abilities (Figs. 4 and 5).

Compared to that of HA-6 and HA-10, the intensity of the negative CD band indicative of n–π* transition of the amide group significantly decreased in the CD spectra of dHA-6 and dHA-10 (Fig. 6). Since 'unsolvated' amides with no attraction and association of water molecules, typically had CD bands at higher wavelengths [24], both dHA-6 and dHA-10 demonstrated a negative CD band centered above 210 nm (Fig. 6), which in part explained a dramatic decrease in the moisture absorptio-retention capacity after HA *N*-deacetylation.

Conclusion

In this study, highly *N*-deacetylated HAs were prepared *via* the NH_2NH_2-HIO_3 procedure and their structures were characterized by HPGPC, ^{1}H NMR and CD. The moisture-absorption (*Ra*) and moisture-retention (*Rh*) abilities of these *N*-deacetylated and re-*N*-acteylated products were compared. Our data demonstrated that the HA *N*-deacetylation resulted in a significant decrease of *R*a and *R*h values. These results were consistent with the appearance of 'unsolvated' amides with no attraction and association of water molecules in the *N*-deacetylated products, as indicated by circular dichroism (CD) spectroscopy. Thus our findings implied that age-induced HA *N*-deacetylation, in addition to the decrease of HA contents in the elderly persons, might account for manifestations of naturally aged skin, such as laxity, sagging, and wrinkling.

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References

1. Weissmann, B., Meyer, K.: The structure of hyalobiuronic acid and of hyaluronic acid from umbilical Cord1,2. J. Am. Chem. Soc. **76** (7), 1753–1757 (1954)
2. Weissmann, B., Meyer, K., Sampson, P., Linker, A.: Isolation of oligosaccharides enzymatically produced from hyaluronic acid. J. Biol. Chem. **208**(1), 417 (1954)
3. Fraser, J.R., Laurent, T.C., Laurent, U.B.: Hyaluronan: its nature, distribution, functions and turnover. J. Intern. Med. **242**(1), 27–33 (1997)

4. Jiang, D., Liang, J., Noble, P.W.: Hyaluronan as an immune regulator in human diseases. Physiol. Rev. **91**(1), 221–264 (2011)
5. Balazs, E.A.: Hyaluronan. From basic science to clinical applications. Vols. 1, 2 and 3. PubMatrix, Edgewater (2011)
6. Juhlin, L.: Hyaluronan in skin. J. Intern. Med. **242**(1), 61–66 (1997)
7. Slevin, M., Kumar, S., Gaffney, J.: Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. J. Biol. Chem. **277**(43), 41046 (2002)
8. Laurent, T.C., Fraser, J.R.: Hyaluronan. FASEB J. **6**(7), 2397–2404 (1992)
9. Manuskiatti, W., Maibach, H.I.: Hyaluronic acid and skin: wound healing and aging. Int. J. Dermatol. **35**(8), 539–544 (1996)
10. Hsu, M.F., Chiang, B.H.: Stimulating effects of Bacillus subtilis natto-fermented Radix astragali on hyaluronic acid production in human skin cells. J. Ethnopharmacol. **125**(3), 474–481 (2009)
11. Longas, M.O., James, D., Lesniak, J., Booth, R.M., McPencow, J.A., Park, J.I.: Hyaluronic acid N-deacetylase assay in whole skin. Biomacromolecules **4**(1), 189–192 (2003)
12. Longas, M.O., Russell, C.S., He, X.Y.: Chemical alterations of hyaluronic acid and dermatan sulfate detected in aging human skin by infrared spectroscopy. Biochim. Biophys. Acta **884**(2), 265–269 (1986)
13. Longas, M.O., Russell, C.S., He, X.Y.: Evidence for structural changes in dermatan sulfate and hyaluronic acid with aging. Carbohydr. Res. **159**(1), 127–136 (1987)
14. Hook, M., Riesenfeld, J., Lindahl, U.: N-[3H]Acetyl-labeling, a convenient method for radiolabeling of glycosaminoglycans. Anal. Biochem. **119**(2), 236–245 (1982)
15. Dahl, L.B., Laurent, T.C., Smedsrod, B.: Preparation of biologically intact radioiodinated hyaluronan of high specific radioactivity: coupling of 125I-tyramine-cellobiose to amino groups after partial N-deacetylation. Anal. Biochem. **175**(2), 397–407 (1988)
16. Baumann, H., Tzianabos, A.O., Brisson, J.R., Kasper, D.L., Jennings, H.J.: Structural elucidation of two capsular polysaccharides from one strain of Bacteroides fragilis using high-resolution NMR spectroscopy. Biochemistry-Us **31**(16), 4081–4089 (1992)
17. Duan, J., Zheng, Y., Dong, Q., Fang, J.: Structural analysis of a pectic polysaccharide from the leaves of Diospyros kaki. Phytochemistry **65**(5), 609–615 (2004)
18. Sun, L., Du, Y., Yang, J., Shi, X., Li, J., Wang, X., Kennedy, J.F.: Conversion of crystal structure of the chitin to facilitate preparation of a 6-carboxychitin with moisture absorption–retention abilities. Carbohydr. Polym. **66**(2), 168–175 (2006)
19. Ying, G.Q., Yang, H., Yi, Y., Xu, F.: Relationships between the molecular structure and moisture-absorption and moisture-retention abilities of succinyl chitosan. Polym. Bull. **59**(4), 509–516 (2007)
20. Yang, S., Guo, Z., Zhou, Y., Zhou, L., Xue, Q., Miao, F., Qin, S.: Synthesis and moisture absorption and retention activities of a carboxymethyl and a quaternary ammonium derivative of alpha, alpha-trehalose. Carbohydr. Res. **345**(1), 120–123 (2010)
21. Yang, S., Guo, Z., Xue, Q., Miao, F., Wang, Y., Qin, S.: The moisture absorption and retention abilities of hyaluronan, chitosan, starch and their quaternary derivatives, pp. 1–4 ICBBE (2009)
22. Oerther, S., Maurin, A.C., Payan, E., Hubert, P., Lapicque, F., Presle, N., Dexheimer, J., Netter, P.: High interaction alginate-hyaluronate associations by hyaluronate deacetylation for the preparation of efficient biomaterials. Biopolymers **54**(4), 273–281 (2000)
23. Cowman, M.K., Matsuoka, S.: Experimental approaches to hyaluronan structure. Carbohyd. Res. **340**(5), 791–809 (2005)
24. Buffington, L.A., Pysh, E.S., Chakrabarti, B., Balazs, E.A.: Far-ultraviolet circular dichroism of N-acetylglucosamine, glucuronic acid, and hyaluronic acid. J. Am. Chem. Soc. **99**(6), 1730–1734 (1977)
25. Hargittai, I., Hargittai, M.: Molecular structure of hyaluronan: an introduction. Struct. Chem. **19**(5), 697–717 (2008)