Synthesis of Novel Supramolecular Assemblies Based on Hyaluronic Acid Derivatives Bearing Bivalent β-Cyclodextrin and Adamantane Moieties

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ABSTRACT: New supramolecular assemblies based on hyaluronic acid stabilized by divalent β-cyclodextrin/adamantane (β-CD/AD) complexes were synthesized with the aim to compare their viscoelastic behavior in aqueous medium with systems resulting from monovalent complexes. For this purpose, dimers of AD and β-CD to be coupled to hyaluronic acid were prepared by multistep chemical synthetic pathways. Investigation of the complexation properties of these dimers by isothermal titration calorimetry (ITC) experiments revealed a pronounced increase of the stability constant. These results can be attributed to divalency as suggested by the value of the complexation enthalpy (ΔH^0) which is twice that of the monovalent complex, in agreement with literature data. Nevertheless, a decrease of the complexation properties of these dimers when fixed on the polysaccharide backbone was observed as a result of an unfavorable entropy of binding. The viscoelastic properties of the supramolecular assemblies were examined by dynamic rheological measurements, showing different behaviors related to the nature and the energy of the interchain physical junctions.

Introduction

Recently, we have described the synthesis and viscoelastic behavior of new supramolecular assemblies based on the monovalent complex formation between β-cyclodextrin (β-CD) and adamantane (AD) molecules, each grafted on chitosan.[1,2] In these assemblies, the "host−guest" complexes were shown to play the role of sticky points (stickers) which, acting as dissipative friction centers, quench drastically the motions of the polysaccharide chain. These sticky points dominate the long time dynamics, which is consequently closely related to the number of stickers per chain, their lifetime, and binding strength. It has been shown that the combination of divalent interactions can be an effective means to increase binding constants between host and guest molecules. Thus, a large number of cyclodextrin dimers showing markedly enhanced binding of ditopic guests compared to the monomeric species have been synthesized.[3−7] From these results, it appeared to us interesting to examine the role of the energy of the junction point on the viscoelastic properties of such temporary networks. For this purpose, we developed novel supramolecular assemblies stabilized by divalent β-CD/AD complexes and compared their viscoelastic behavior in aqueous media with systems resulting from monovalent complexes (see Figure 1). The formation of divalent complexes of β-CD and their thermodynamic properties have already been studied in aqueous solution[3b,d,e,4a] and have also been used for the construction of self-assembled monolayers of molecules (SAMs).[8−10] In this work, we use specific divalent complexes of β-CD for the development of higher order multivalent interactions between polysaccharide chains in solution.

The polymer used for this study is hyaluronic acid (HA), a linear polysaccharide composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid (Figure 2). It is the only nonsulfated glycosaminoglycan found in the extracellular matrix. Because of its unique viscoelastic properties and biological performance, HA has become an attractive building block for the development of new biocompatible materials with many applications in viscosupplementation, tissue engineering, and drug delivery.[11,12]

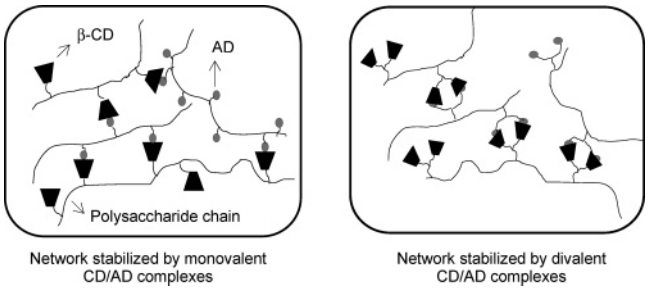

Figure 1. Schematic representation of the targeted physical networks in which monovalent and divalent β-CD/adamantane complexes play the role of junction points.

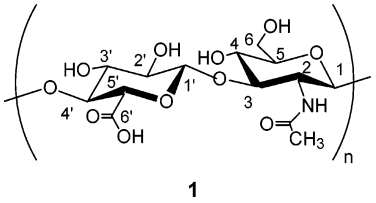

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Figure 2. Chemical structure of hyaluronic acid.

In the first section of this paper, we describe the synthesis of new dimers of β-cyclodextrin and adamantane and compare their binding properties with the monomeric species. In the second section, we report on the coupling of these dimers with hyaluronic acid and the binding properties of the resulting host and guest polymers derived from isothermal titration calorimetry (ITC). In the last section, the role of the binding strength of CD complexes on the viscoelastic properties of the networks is examined.

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Experimental Section

Materials. Bacterial hyaluronic acid, in the form of the sodium salt, was obtained from ARD (Pomacle, France). The molecular weight distribution and the weight-average molecular weight were determined by size exclusion chromatography using a Waters GPCV Alliance 2000 chromatograph equipped with three on-line detectors: a differential refractometer, a viscometer, and a light scattering detector (MALLS) from Wyatt; the solution was injected at a concentration of 5×10^{-4} g/mL in 0.1 M $NaNO_3$. The polymolecularity of the sample is $M_w/M_n \sim 1.5$. The weight-average molecular weight was determined to be 3×10^5 g/mol. The overlap concentration C^* for this HA sample in 0.1 M NaCl at 25 °C is around 1.3 g/L. This value was deduced from the intrinsic viscosity,[13] assuming that $C^*[\eta]$ is about unity.[14] The β-cyclodextrin (β-CD) was kindly supplied by Roquette Frères (Lestrem, France). Adamantane acetaldehyde **25** was synthesized in the laboratory as described previously.[2] The β-CD monocarboxylic acid **10** and β-CD aldehyde **21** derivatives were obtained from natural β-CD following synthetic methodologies reported elswhere.[15] All other chemical products and reagents were purchased from Fluka (Buchs, Switzerland).

NMR Spectroscopy. ^{1}H NMR experiments were performed using Bruker DRX400 and AC300 spectrometers operating at 400 and 300 MHz, respectively. ^{13}C NMR spectra were recorded with Bruker DRX400 and AC300 spectrometers operating at 100 and 75 MHz, respectively. Chemical shifts (δ in ppm) are given relative to external tetramethylsilane (TMS = 0 ppm), and calibration was performed using the signal of the residual protons of the solvent as a secondary reference. Deuteriun oxide and deutered chloroform were obtained from SDS (Vitry, France). Details concerning experimental conditions are given in the figure captions.

Mass Spectrometry. Electrospray mass spectra were measured in the positive mode on a ZabSpec TOF (Micromass, UK) mass spectrometer. Poly(ethylene glycol) derivatives, used for external

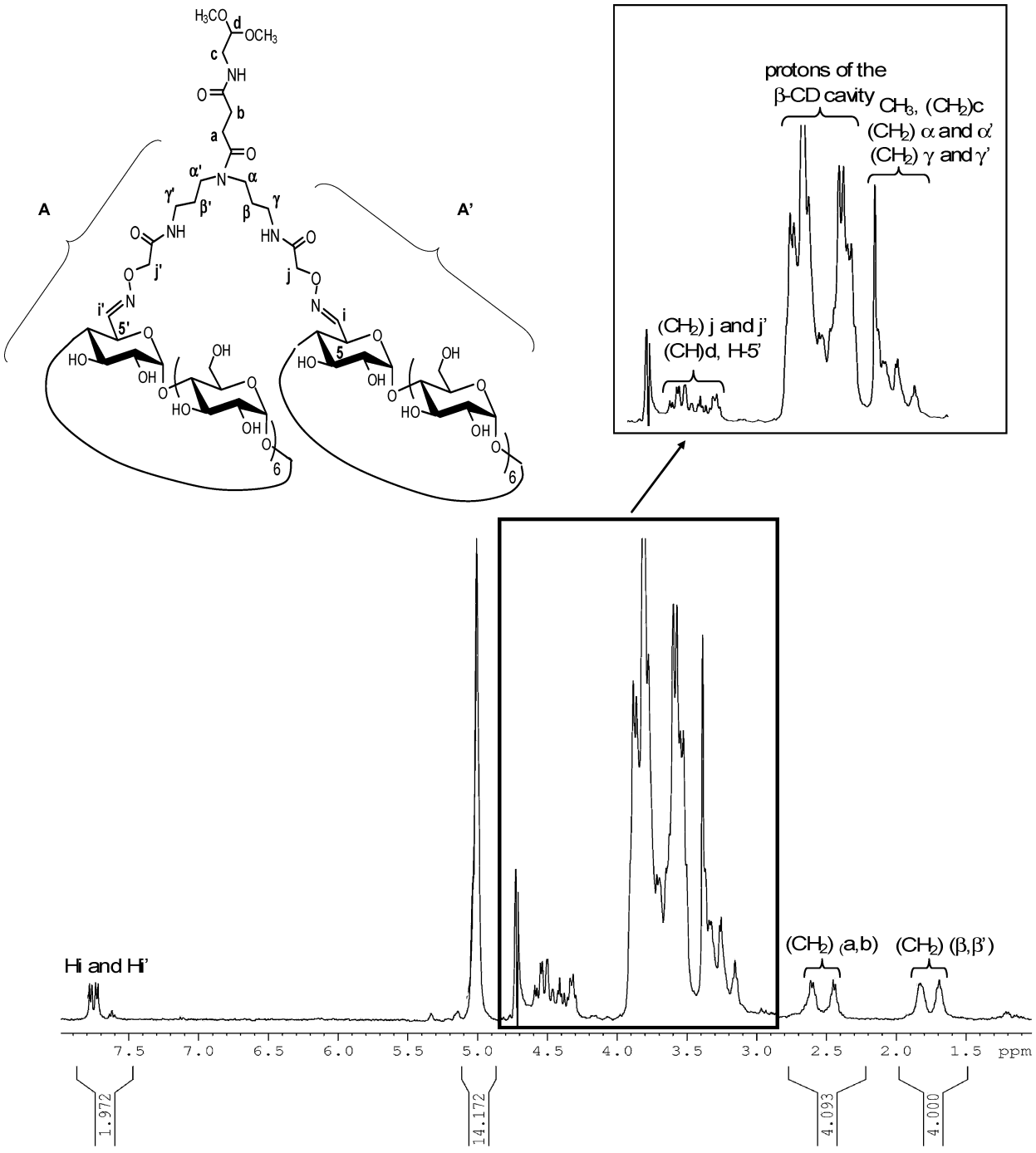


Figure 3. ^{1}H NMR spectrum (D_2O, 400 MHz, 25 °C, 6 mg/mL) of the acetalic dimer of β-CD **11**.

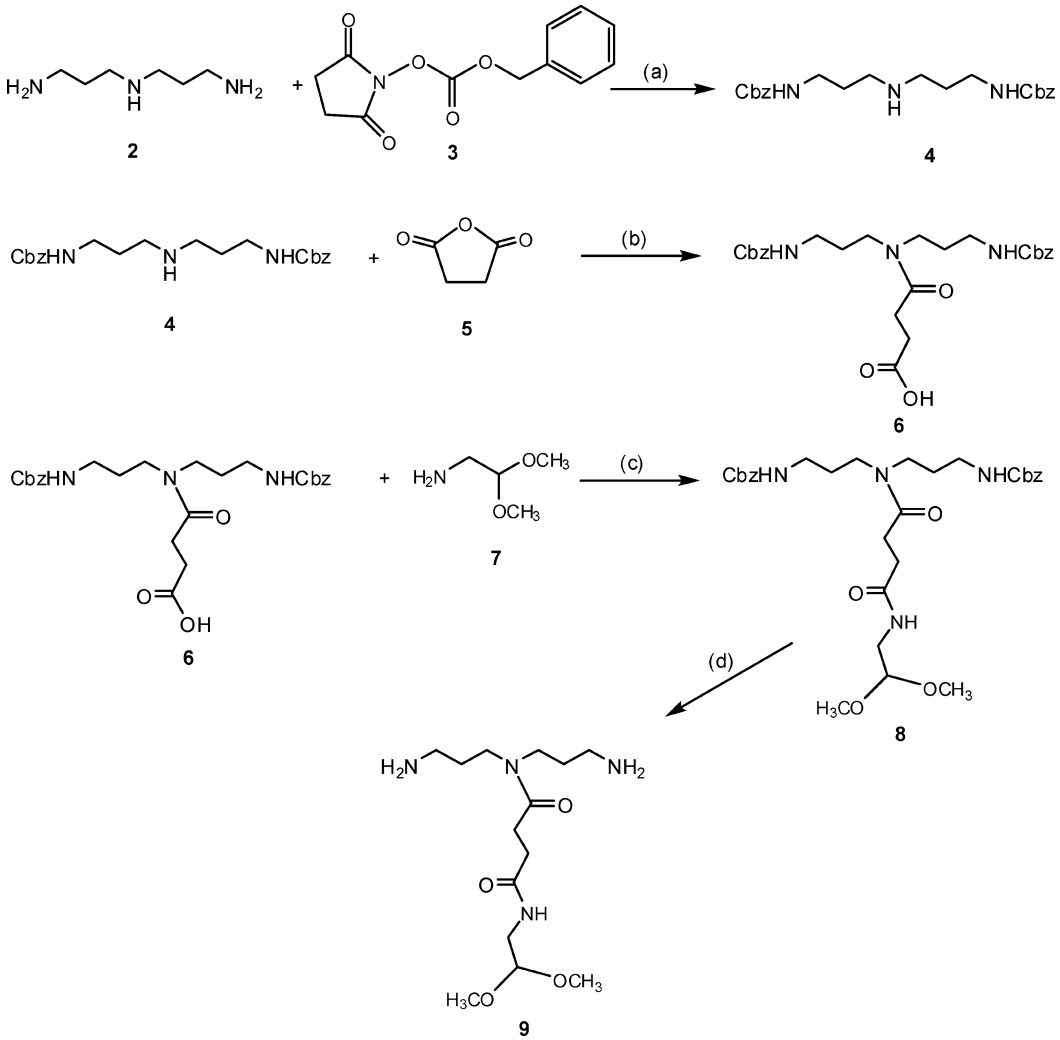
calibration, were dissolved in methanol/water (1:1 (v/v)) at a concentration of 0.1 mg/mL and infused into the electrospray ion source. The capillary voltage was set to 4 kV.

Titration Calorimetry. Isothermal titration calorimetry measurements of the dimer−dimer and dimer−polymer interactions were performed using a model 4200 microcalorimeter from Calorimetry Sciences Corp. (Lindon, UT) or a Microcal VP-ITC titration microcalorimeter (Northampton, MA). The high viscosity of the mixtures of the host and guest HA derivatives precluded ITC measurements of polymer−polymer interactions. In individual titrations, injections of 10 μL of ligand (sodium adamantane acetate (ADAc), dimer **18**, dimer **11**) were added from the computer-controlled 250 μL microsyringe at an interval of 5 min into the receptor solution (β-CD, dimer **11**, HA-$(CD)_2$ **22**, HA-$(AD)_2$ **26** (cell volume = 1.3 or 1.4478 mL) containing the same solvent as the solution of ligand (pure water, 0.025 or 0.2 M NaCl), while stirring at 297 rpm at 25 °C. The observed heat effects under identical injections of ligand into a cell containing only the solvent were identical to the heat signals at the end of titration, after the saturation was reached. The raw experimental data were presented as the amount of heat produced following each injection of ligand as a function of time. The amount of heat produced per injection was calculated by integration of the area under individual peaks by the instrument software, after taking into account heat of dilution. The experimental data were fitted to a theoretical titration curve using the instrument software (Bindworks program (CSC) or ORIGIN software (Microcal)), refining the enthalpy change (in kJ/mol), ΔH^0, the association constant (in L/mol), K_a, and the stoichiometry of the interaction (number of binding sites per receptor),[16] n. In all cases, calculations were performed using the "one set of binding sites" model. Concerning the ITC experiments performed on the AD and the CD dimers, the concentrations of the host and guest considered for the calculations are the concentrations of the dimers and not those of the β-CD and AD molecules. Consequently, the value of the stoichiometry n corresponds to the binding mode of the dimers. It should be noted that the stoichiometry of the interaction can be directly read on the thermogram. For example, if a receptor has one binding site, the equivalence point of the titration will be at a molar ratio of 1:1. On the other hand, if it has two binding sites, the equivalence point of the titration will be at a molar ratio of 2:1.[16]

Rheological Experiments. Oscillatory experiments were performed with a cone−plate rheometer (AR1000 from TA Instruments). All the dynamic rheological data were checked as a function of strain amplitude to ensure that the measurements were performed in the linear viscoelastic region. The cone used has a diameter of 4 cm and an angle of 3°59′. Experiments were carried out at 25 °C, with a film of silicone to avoid solvent evaporation. HA-(CD), $HA(CD)_2$, HA(AD), and $HA(AD)_2$ were dissolved in aqueous 0.025 M NaCl. Salt was added in order to screen the long-range electrostatic repulsions between negatively charged chains. The dissolution time was at least 12 h at room temperature. The solutions of CD- and AD-grafted HA samples were then mixed. Gel-like samples were vigorously stirred and allowed to rest for at least 1 h at room temperature. We checked that the rheological properties of the samples did not change with time (from 1 to 24 h).

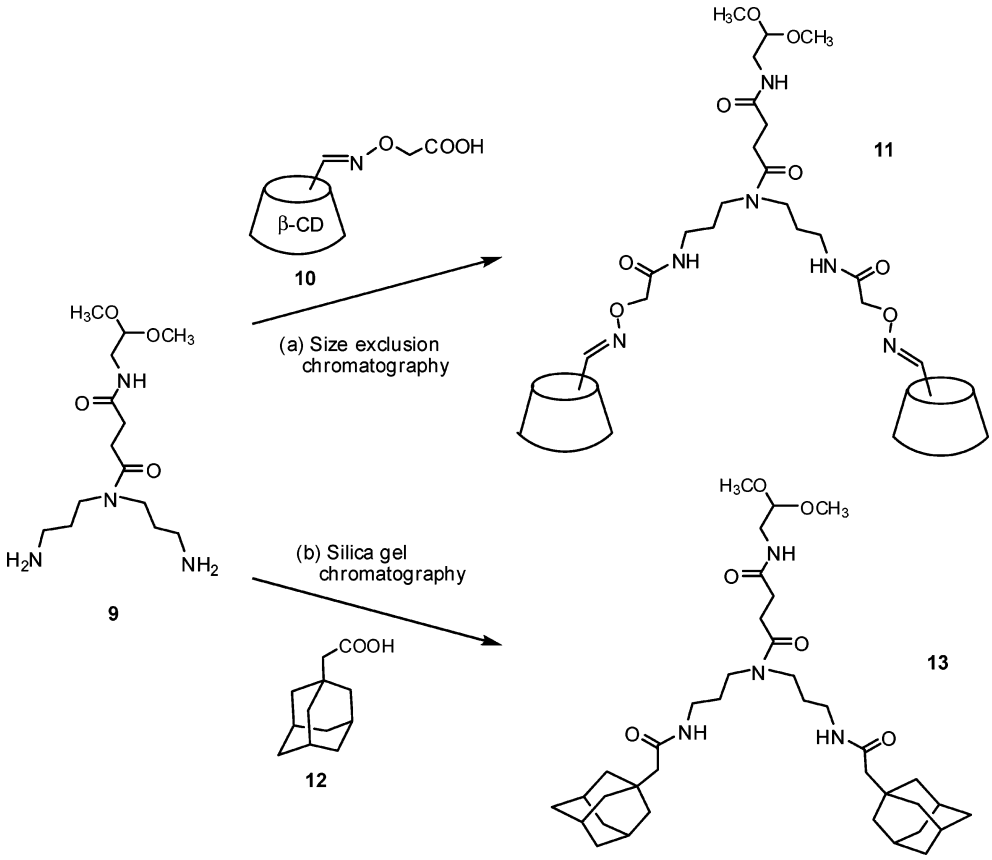
Synthesis. Reactions for the synthesis of the dimeric species were monitored by thin-layer chromatography (TLC) on a precoated plate

Scheme 1. Synthesis of the Common Intermediate 9[a]



[a] Conditions: (a) CH_2Cl_2, RT, 48 h; (b) pyridine, CH_2Cl_2, RT, 1 h; (c) NEt_3, HOBt, DCC, DMF, RT, 48 h; (d) Pd/C, H_2, RT, 3 h.

Scheme 2. Synthesis of the Dimers of β-CD 11 and AD 13[a]



[a] Conditions: (a) DIC, HOBt, DMF, RT, 24 h; (b) Et_3N, DCC, HOBt, DMF RT, 48 h.

of silica gel 60 F_{254} (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detection by charring with a 10% (v/v) ethanolic solution of sulfuric acid or an ethanolic solution of phosphomolybdic acid (50 g/L). Flash column chromatography was performed on silica gel (Merck Gerduran SI 60, 40-63) with indicated eluant. The letters used for the assignment of the ^1H and ^{13}C NMR signals for the dimeric species are indicated in Figure 3.

N^1,N^9-Bis(carbobenzyloxy)-N^5-(succinylamido)norspermidine ***6***. To a solution of norspermidine, **2** (2 g, 15.2 mmol), under nitrogen in dry dichloromethane (CH_2Cl_2, 165 mL), *N*-(benzyloxycarbonyloxy)succinimide, **3** (7 g, 30 mmol), was added in powder form. The resulting mixture was stirred under nitrogen at room temperature for 48 h. The mixture was washed five times with water to remove *N*-hydroxysuccinimide (NHS) formed during the reaction. The organic phase was dried on sodium sulfate and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 7:3 (v/v)) to give compound **4** as a white powder in 40% yield (3.4 g, 9 mmol).

To a solution of **4** (1.69 g, 4.23 mmol) under nitrogen in dry CH_2Cl_2 (60 mL), pyridine (0.374 mL, 4.66 mmol) and succinic anhydride, **5** (0.466 g, 4.66 mmol), were added. After stirring under nitrogen at room temperature for 1 h, the mixture was successively washed with a 0.1 M HCl solution, water, an aqueous solution of sodium bicarbonate, and again with water. The organic phase was dried on sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 9:1 (v/v)) to give compound **6** as a white solid in 80% yield (1.68 g, 3.36 mmol).

R_f = 0.41 (CH_2Cl_2/MeOH 9:1 (v/v)). ^1H NMR (400 MHz, $CDCl_3$): δ 7.3 (m, 10H, 2 Ph), 5.52 (s, 2H, Cbz−N*H*), 5.05 (m, 4H, 2 (Ph−CH_2)), 3.41 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 3.3 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 3.25 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 3.1 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 2.51 (m, 2H, $(CH_2)_a$), 2.53 (m, 2H, $(CH_2)_b$), 1.81 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.68 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$)).

^{13}C NMR (100 MHz, $CDCl_3$): δ 177.4, 173.7, 158.1 (4 C═O), 136.7, 129.2−130 (12 C, 2 Ph), 67.8, 68.2 (2C, 2 (*C*H_2−Ph)), 46.9, 44.2 (2C, 2 $(CH_2)_{\alpha \text{ and } \alpha'}$), 39.8, 39 (2C, 2 $(CH_2)_{\gamma \text{ and } \gamma'}$), 30.9, 29 (2C, $(CH_2)_{a \text{ et } b}$), 30.3, 29 (2C, $(CH_2)_{\beta \text{ and } \beta'}$).

ESI-HRMS: $[M + Na]^+$ *m/z* 522.22162 Calcd for $C_{26}H_{33}N_3O_7$-Na; Found 522.2214. $[M + K]^+$ *m/z* 538.19556 Calcd for $C_{26}H_{33}N_3O_7K$; Found 538.1930. $[M-H + 2Na]^+$ *m/z* 544.20357 Calcd for $C_{26}H_{32}N_3O_7Na_2$; Found 544.2033.

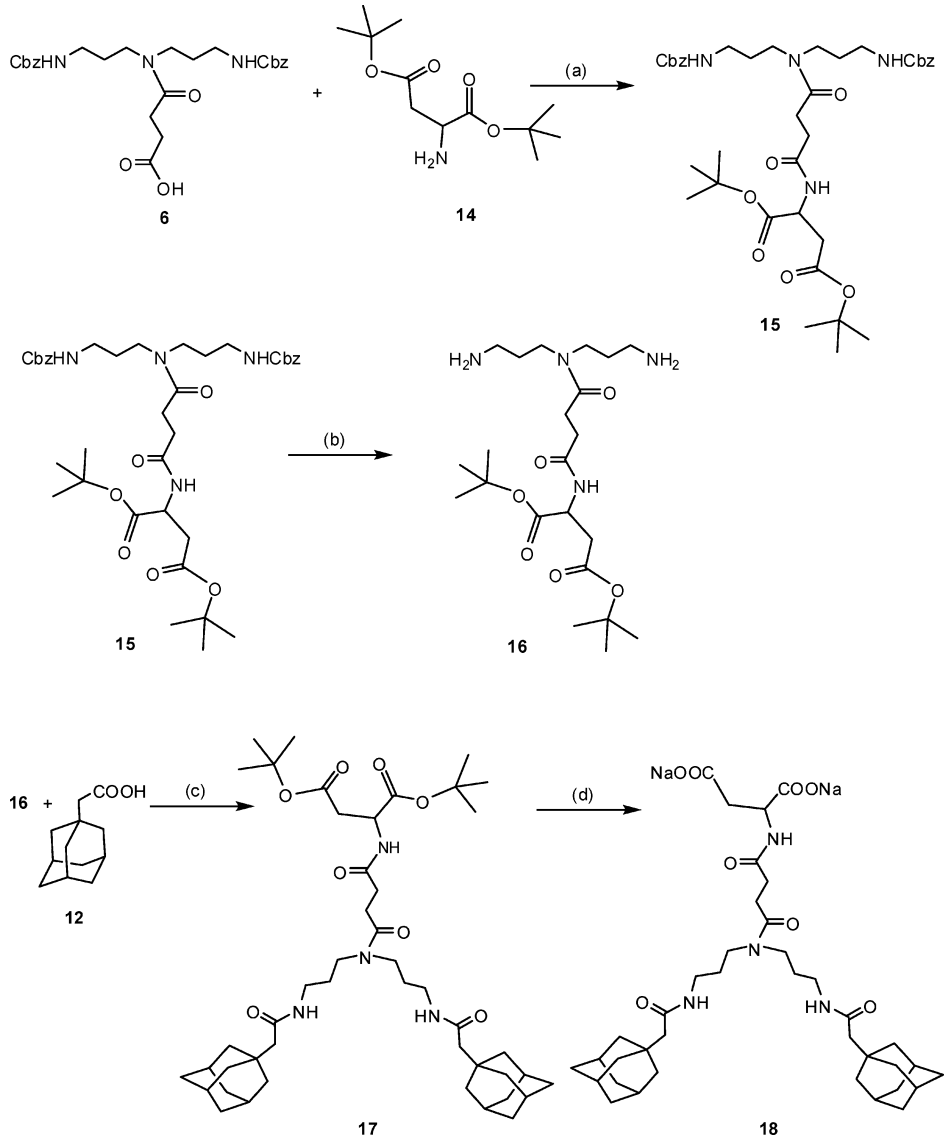
N^1,N^9-Bis(carbobenzyloxy)-N^5-(N-(2,2-dimethoxyethylamido)succinylamido)norspermidine ***8***. To a solution of **6** (3.19 g, 6.39 mmol) under nitrogen in dry DMF (100 mL), *N*-hydroxybenzotriazole (0.861 g, 6.39 mmol), *N*,*N*′-dicyclohexylcarbodiimide (1.58 g, 7.67 mmol,), triethylamine (0.88 mL, 0.645 g), and 2,2-dimethoxyethylamine **7** (0.671 g, 6.39 mmol) were successively added. The resulting mixture was stirred under nitrogen at room temperature for 48 h. After evaporation of DMF, the residue was dispersed in diethyl ether. A precipitate of dicyclohexylurea (DCU) was formed as a brown paste and was removed by filtration. The filtrate was washed with a 0.1 M HCl solution, water, a 0.1 M NaOH solution, and again with water. The organic phase was dried on sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 97:3 (v/v)) to give compound **8** as a colorless oil in 51% yield (1.87 g, 3.19 mmol).

R_f = 0.41 (CH_2Cl_2/MeOH 97:3 (v/v)). ^1H NMR (400 MHz, $CDCl_3$): δ 7.3 (m, 10H, 2 Ph), 6 (s,1H, Cbz−N*H*), 5.7 (s,1H, N*H*-Cbz), 5.1 (m, 4H, 2 (Ph−CH_2)), 4.35 (t, 1H, J = 5.6 Hz, $(CH)_d$−$(OCH_3)_2$), 3.3−3.45 (m, 12 H, (−O(CH_3)$_2$, $(CH_2)_{\alpha \text{ and } \alpha'}$, $(CH_2)_C$−NHCO), 3.21 (m, 2H, $(CH_2)_{\gamma \text{ and } \gamma'}$), 3.09 (m, 2H, $(CH_2)_{\gamma \text{ and } \gamma'}$), 2.61 (m, 2H, $(CH_2)_a$), 2.51 (m, 2H, $(CH_2)_b$), 1.81 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.62 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.5, 156.5, (4C, 4C═O), 136, 137, 129−127 (12C, 2Ph), 102.5 (1C, $(CH)_d$−$(OCH_3)_2$), 67, 66.5 (2C, 2(*C*H_2−Ph)), 54.2 (2C, 2(CH_3)), 45, 42.3 (2C, 2$(CH_2)_{\alpha \text{ and } \alpha'}$), 41 (1C, $(CH_2)_c$−NHCO), 38.5, 37.5 (2C, $(CH_2)_{\gamma \text{ and } \gamma'}$), 31.5, 28 (2C, $(CH_2)_{a \text{ and } b}$), 29, 27.5 (2C, $(CH_2)_{\beta \text{ and } \beta'}$).

ESI-HRMS: $[M + Na]^+$ *m/z* 609.29003 Calcd for $C_{30}H_{42}N_4O_8$-Na; Found 609.2898, $[M + K]^+$ *m/z* 625.26397 Calcd for $C_{30}H_{42}N_4O_8K$; Found 625.2646.

Scheme 3. Synthesis of the Water-Soluble Dimer of AD 18[a]



[a] Conditions: (a) NEt_3, HOBt, DCC, DMF, RT, 36 h; (b) Pd/C, H_2, MeOH, RT, 3 h; (c) NEt_3, HOBt, DCC, DMF, RT, 36 h; (d) CH_2Cl_2/TFA, then NaOH, RT, 12 h.

N^5-(N-(2,2-Dimethoxyethyl)succinylamido)norspermidine ***9***. To a solution of **8** (1 g, 1.7 mmol) in MeOH (100 mL), activated palladium on carbon (160 mg) was added. The resulting mixture was stirred under a hydrogen atmosphere at room temperature for 3 h. The mixture was then filtered on celite, and the filtrate was evaporated under reduced pressure to give coumpound **9** in 79% yield (0.422 g, 1.07 mmol).

ESI-HRMS: $[M + Na]^+$ *m*/*z* 341.21648 Calcd for $C_{14}H_{30}N_4O_4$-Na; Found 341.2163. $[M + H]^+$ *m*/*z* 319.23453 Calcd for $C_{14}H_{31}N_4O_4$; Found 319.2344.

R_f = 0.41 (CH_2Cl_2/MeOH 97:3 (v/v)). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.35 (t, 1H, J = 5.6 Hz, (C*H*)$_d$−(OCH$_3$)$_2$), 3.3−3.45 (m, 12 H, (−O(C*H*$_3$)$_2$, (CH$_2$)$_{\alpha \text{ and } \alpha'}$, (C*H*$_2$)$_c$−NHCO), 3.21 (m, 2H, (CH$_2$)$_{\gamma \text{ and } \gamma'}$), 3.09 (m, 2H, (CH$_2$)$_{\gamma \text{ and } \gamma'}$), 2.61 (m, 2H, (C*H*$_2$)$_a$), 2.51 (m, 2H, (C*H*$_2$)$_b$), 1.81 (m, 2H, (CH$_2$)$_{\beta \text{ or } \beta'}$), 1.62 (m, 2H, (CH$_2$)$_{\beta \text{ or } \beta'}$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.5, 156.5, (2C, 4C═O), 102.5 (1C, (*C*H)$_d$−(OCH$_3$)$_2$), 67, 54.2 (2C, 2(CH$_3$)), 45, 42.3 (2C, 2(CH$_2$)$_{\alpha \text{ and } \alpha'}$), 41 (1C, (CH$_2$)$_c$−NHCO), 38.5, 37.5 (2C, (CH$_2$)$_{\gamma \text{ and } \gamma'}$), 31.5, 28 (2C, (CH$_2$)$_{a \text{ and } b}$), 29, 27.5 (2C, (CH$_2$)$_{\beta \text{ and } \beta'}$).

Acetalic Dimer of β-CD ***11*****.** To a solution of β-CD monoacid[15] **10** (1.12 g, 0.944 mmol) under nitrogen in dry DMF (90 mL), *N*-hydroxybenzotriazole (0.255 g, 1.88 mmol), *N*,*N*′-diisopropylcarbodiimide (0.475 g, 3.77 mmol), and compound **9** (0.136 g, 0.429 mmol), each dissolved in DMF, were successively added. The resulting mixture was stirred at room temperature for 24 h. After evaporation of most of the solvent, the residual syrup was poured into acetone (400 mL). The crude product was isolated by filtration and dissolved again in DMSO (15 mL) to remove soluble impurities by reprecipitation with acetone. After filtration, the product was dried under reduced pressure at 25 °C for 12 h. The precipitate dissolved in water was purified by size exclusion chromatography on a Biogel (Bio-Rad) P-6 column with water as the eluant, at room temperature. The flow rate was 80 mL/h. Fractions were monitored by a differential refractometer. The product was then recovered by freeze-drying as a white powder in 67% yield (0.649 g, 0.287 mmol).

^{1}H NMR (400 MHz, D_2O): δ 7.76 (d, 1H, J = 6.7 Hz, (CH)$_i$═NO), 7.71 (d, 1H, J = 6.7 Hz, (CH)$_{i'}$═NO), 5.05−4.95 (m, 14 H, H-1and H-1), 4.53 (m, 2H, (CH$_2$)$_j$−ON), 4.48 (m, 2H, (CH$_2$)$_{j'}$−ON), 4.41 (t, 1H, J = 5.6 Hz, (C*H*)$_d$−(OCH$_3$)$_2$), 4.32 (m, H-5′), 3.9−3.4 (m, cyclodextrin protons), 3.37 (s, 6H, 2(CH$_3$)), 3.35 (m, 4H, (CH$_2$) $_{\alpha \text{ or } \alpha'}$ and (CH$_2$)$_c$−NHCO), 3.31 (m, 2H, (CH$_2$) $_{\alpha \text{ or } \alpha'}$), 3.25 (m, 2H, (CH$_2$)$_{\gamma \text{ or } \gamma'}$), 3.1 (m, 2H, (CH$_2$)$_{\gamma \text{ or } \gamma'}$), 2.59 (m, 2H, (CH$_2$)$_a$−CO), 2.44 (m, 2H, (CH$_2$)$_b$−CO), 1.81 (m, 2H, (CH$_2$)$_{\beta \text{ or } \beta'}$), 1.68 (m, 2H, (CH$_2$)$_{\beta \text{ or } \beta'}$).

^{13}C NMR (100 MHz, D_2O): δ 174.9, 174.1, 171.9, 171.6 (4C, 4C═O), 151.1, 151.2 (2C, (*C*H)$_{i \text{ and } i'}$═NO), 102.7 (1C, (*C*H)$_d$−

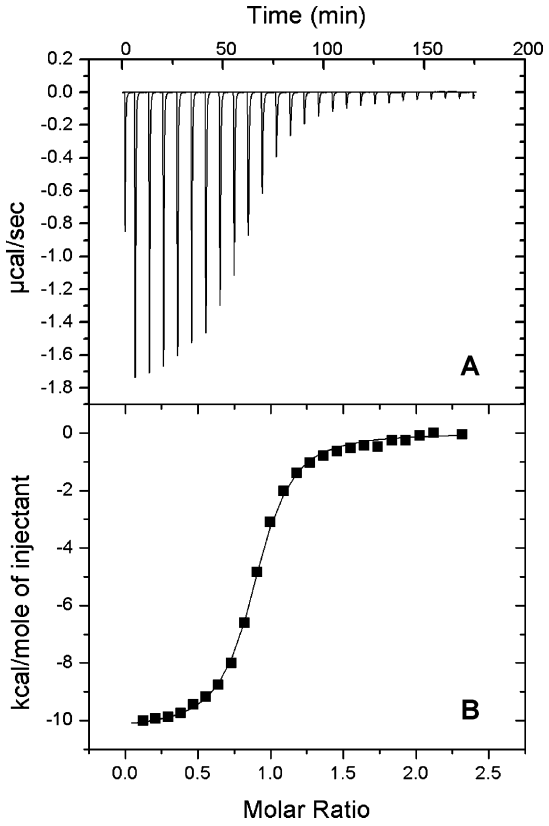


Figure 4. Calorimetric titration of the CD dimer **11** with the AD dimer **18** in 0.025 NaCl at 25 °C (experiment b in Table 1). (A) Raw data for 30 sequential injections (10 μL per injection) of AD dimer ([AD dimer] = 0.569 mM) injected into CD dimer solution ([CD dimer] = 0.047 mM). (B) The integrated curve showing experimental points and the best fit for titration of CD dimer with AD dimer.

$(OCH_3)_2$), 102.5−101.7 (C-1, C-1′), 82.9 (C-4′), 81.5, 80.8 (C-4), 73.4, 73.1, 72.4, 72.2, 71.9 (C-3, C-3′, C-5, C-2, C-2′), 72.4 (2C, $(CH_2)_{j \text{ and } j'}$−ON), 68.9 (C-5′), 54.7 (2(CH_3), 43.4, 41.4 (2C, $(CH_2)_{\alpha \text{ and } \alpha'}$), 45.6 (1C, $(CH_2)_c$−NHCO), 36.8, 36.7 (2C, $(CH_2)_{\gamma \text{ and } \gamma'}$)), 31, 28.3 (2C, $(CH_2)_{a \text{ and } b}$-CO), 27.9, 26.8 (2C, $(CH_2)_{\beta \text{ and } \beta'}$).

ESI-HRMS: $[M + 2Na]^+$ *m/z* 1369.4631 Calcd for $C_{102}H_{168}N_6O_{76}Na_2$; Found 1369.4640. $[M + Na + K]^{2+}$ *m/z* 1377.4500 Calcd for $C_{102}H_{168}N_6O_{76}NaK$; Found 1377.4593.

*Acetalic Dimer of AD **13***. To a solution of (1-adamantyl)acetic acid, **12** (0.738 g, 3.8 mmol), under nitrogen in dry DMF (99 mL), *N*-hydroxybenzotriazole (0.514 g, 3.8 mmol), *N*,*N*′-dicyclohexylcarbodiimide (0.941 g, 4.56 mmol), triethylamine (0.192 g, 1.9 mmol), and compound **9** (0.55 g, 1.73 mmol), each dissolved in DMF, were successively added. The resulting mixture was stirred at room temperature for 48 h. After evaporation of DMF, the residue was dispersed in diethyl ether. A precipitate of DCU was formed as a brown paste and was removed by filtration. The filtrate was washed with a solution of a 0.3 M NaOH solution, water, a 0.1 M HCl solution, and again water. The organic phase was dried on sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 95:5 (v/v)) to give compound **13** as an oil in 43% yield (0.498 mg, 0.0744 mmol).

R_f = 0.3 (CH_2Cl_2/MeOH 9:1 (v/v)). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.55 (m, 1H, NH), 6.28 (m, 1H, NH), 6.03 (m, 1H, NH), 4.40 (t, 1H, J = 6.1 Hz, $(CH)_d$−$(OCH_3)_2$), 3.48−3.26 (12 H, 2 (CH_3), $(CH_2)_{\alpha \text{ and } \alpha'}$, $(CH_2)_{\gamma \text{ or } \gamma'}$ ($CH_2)_c$−NHCO), 3.17 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 2.69 (m, $(CH_2)_a$−CO), 2.59 (m, $(CH_2)_b$−CO), 2−1.94 (8H, H (adamantane), $(CH_2)_{\beta \text{ or } \beta'}$), 1.87 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.78−1.60 (28 H, H (adamantane)).

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7, 173.6, 172.8, 172.4, 137 (4C, 4C═O), 103.4 (1C, $(CH)_d$−$(OCH_3)_2$), 55.4 (6C, 2(CH_3)), 46.6 (1C, $(CH_2)_c$−NHCO), 43.6, 42.3 (2C, $(CH_2)_{\alpha \text{ and } \alpha'}$), 38.1, 36.9 (2C, $(CH_2)_{\gamma \text{ and } \gamma'}$), 32.3, 29.9 (2C, $(CH_2)_{a \text{ and } b}$−CO), 52.9, 44.4−43.1, 38.8−37.2, 28.5 (C (adamantane), CH_2−adamantane).

ESI-HRMS: $[M + Na]^+$ *m/z* 693.45671 Calcd for $C_{38}H_{62}N_4O_6Na$; Found 693.4553. $[M + K]^+$ *m/z* 709.43064 Calcd for $C_{38}H_{62}N_4O_6K$; Found 709.4259.

N^1,*N*^{9}*-Bis(carbobenzyloxy)-N*^{5}*-(N-(*L*-di-tert-butyl ester aspartamido)succinylamido)norspermidine **15***. To a solution of **6** (2 g, 4 mmol) under nitrogen in dry DMF (30 mL), *N*-hydroxybenzotriazole (0.54 g, 4 mmol), *N*,*N*′-dicyclohexylcarbodiimide (0.99 g, 4.8 mmol) dissolved in DMF, and L-aspartic acid di-*tert*-butyl ester hydrochloride, **14** (0.981 g, 4 mmol), dissolved in DMF containing triethylamine (0.401 g, 4 mmol) were successively added. The resulting mixture was stirred at room temperature for 36 h. After evaporation of DMF, the residue was dispersed in CH_2Cl_2. A precipitate of DCU was formed as a brown paste and was removed by filtration. The filtrate was washed with a 0.1 M HCl solution, water, an aqueous solution of sodium bicarbonate, and again water. The organic phase was dried on sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 98:2 (v/v)) to give compound **15** as an oil in 84% yield (2.44 g, 3.36 mmol).

R_f = 0.42 (CH_2Cl_2/MeOH 95:5 (v/v)). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45−7.3 (m, 10H, 2Ph), 5.1 (m, 4H, 2CH_2−Ph), 4.65 (m, 1H, C*H*−COO), 3.39 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 3.31 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 3.22 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 3.11 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 2.84 (dd, 2H, J = 4.19 Hz, CH_2−COO), 2.62 (m, 2H, $(CH_2)_a$−CO), 2.55 (m, 2H, $(CH_2)_b$−CO), 1.93 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.82 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.48 (18H, 6 (CH_3)).

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.5, 173.3, 171.6, 171.1 (4C, (C═O)), 129.9−129.1 (12 C, 2Ph), 68.05, 67.75 (2C, 2(CH_2−Ph)), 50.4 (1C, (*C*H)−COO), 46.2, 43.6 (2C, $(CH_2)_{\alpha \text{ and } \alpha'}$), 39.8, 39 (2C, $(CH_2)_{\gamma \text{ and } \gamma'}$), 38.7 (1C, (CH_2)−COO), 32.3, 29 (2C, $(CH_2)_{a \text{ and } b}$−CO), 30.2, 28.9 (2C, $(CH_2)_{\beta \text{ and } \beta'}$), 29.3−28.2 (6C, 6$(CH_3)$).

ESI-HRMS: $[M + Na]^+$ *m/z* 749.37376 Calcd for $C_{38}H_{54}N_4O_{10}Na$; Found 749.3746. $[M + K]^+$ *m/z* 765.34770 Calcd for $C_{38}H_{54}N_4O_{10}K$; Found 765.3459.

N^1,*N*^{9}*-Bis(adamantylamido)-N*^{5}*-((*L*-di-tert-butyl ester aspartamido)succinylamido)norspermidine* (***17***). Compound **17** was synthesized from **15** according to reaction conditions similar to those used for the synthesis of **13** from **8**. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/MeOH 97:3 (v/v)) affording **17** as an oil in 53% yield (1.36 mg, 1.68 mmol).

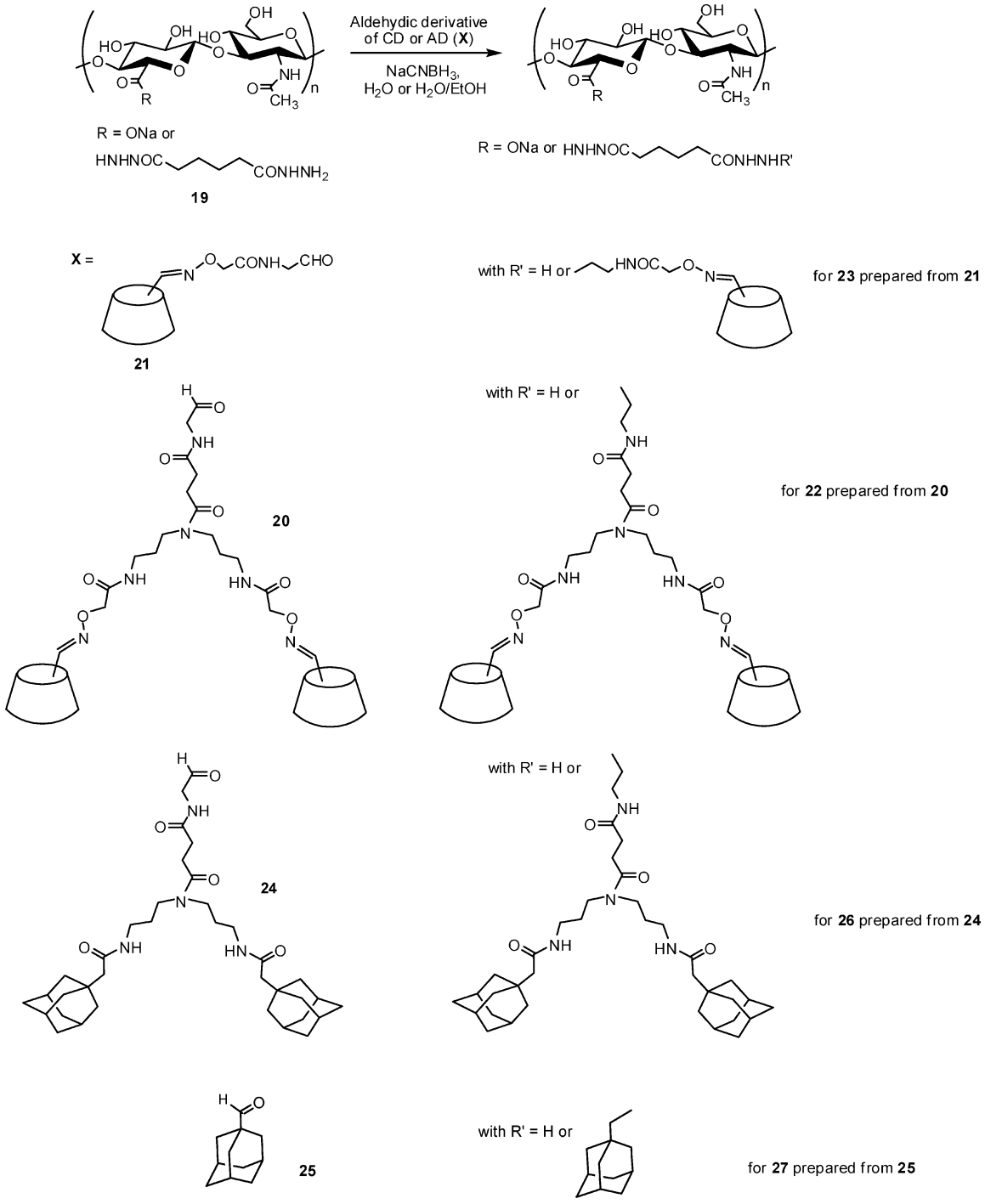
R_f = 0.41 (CH_2Cl_2/MeOH 95:5 (v/v)). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.65 (m, 1H, C*H*−COO), 3.43 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 3.35 (m, 2H, $(CH_2)_{\alpha \text{ or } \alpha'}$), 3.27 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 3.14 (m, 2H, $(CH_2)_{\gamma \text{ or } \gamma'}$), 2.84 (dd, 2H, J = 4.19 Hz, CH_2−COO), 2.62 (m, 2H, $(CH_2)_a$−CO), 2.55 (m, 2H, $(CH_2)_b$−CO), 1.99−1.91 (8H, H (adamantane), $(CH_2)_{\beta \text{ or } \beta'}$), 1.85 (m, 2H, $(CH_2)_{\beta \text{ or } \beta'}$), 1.59−1.74 (28H, H (adamantane)), 1.48 (18H, 6(CH_3)).

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.5, 173.2, 172.8, 172.4, 171.6, 170.9 (6C═O), 53, 52.8 (2C, CH_2−adamantane)), 46.2, 43.5 (2C, $(CH_2)_{\alpha \text{ and } \alpha'}$)), 44.2−43.8 (C adamantane), 38.8 (1C, CH_2−COO), 38.1 (C adamantane, $(CH_2)_{\gamma \text{ or } \gamma'}$), 36.9 (1C, $(CH_2)_{\gamma \text{ or } \gamma'}$),

Table 1. Thermodynamic Parameters for Inclusion Complex Formation of Sodium Adamantane Acetate (ADAc) with Natural β-CD (Experiment a) and of the AD Dimer 18 with the CD Dimer 11 (Experiment b), Derived from Calorimetric Titration Experiments in Pure Water at 25 °C

expt	CD derivative	[CD monomer] or [CD dimer] (mM)	[AD monomer] or [AD dimer] (mM)	$K_a \times 10^{-6}$ (M^{-1})	ΔH^0 (kJ/mol)	$T\Delta S^0$ (kJ/mol)	*n* (*n*AD:1CD derivative)
a	natural β-CD	0.798	8.81	0.075 ± 0.09	−25.8 ± 1.2	2.00 ± 1.13	0.90 ± 0.02
b	CD dimer **11**	0.047	0.569	1.14 ± 0.09	−43.1 ± 1.1	−8.55 ± 0.98	0.90 ± 0.08

Scheme 4. Synthesis of HA Derivatives Bearing Pendent Dimeric or Monomeric CD and AD Molecules



33.7 (1C, *C*H−COO), 32.2, 29.1 (2C, $(CH_2)_{a\ and\ b}$−CO)), 30−29.8 (C adamantane, $(CH_2)_{\beta\ and\ \beta'}$), 29.4−29.2 (6C, 6($CH_3$)), 28.7 (C adamantane).

ESI-HRMS: $[M + Na]^+$ *m*/*z* 833.54044 Calcd for $C_{46}H_{74}N_4O_8$-Na; Found 833.5399. $[M + K]^+$ *m*/*z* 849.51437 Calcd for $C_{46}H_{74}N_4O_8K$; Found 849.5183.

Water-Soluble Dimer of AD ***18.*** Compound **17** (1.33, 1.64 mmol) was dissolved in CH_2Cl_2/trifluoroacetic acid (TFA) (5:5 (v/v)). The resulting mixture was stirred at room temperature for 1 h. After evaporation of the CH_2Cl_2/TFA mixture, the residue dissolved in CH_2Cl_2 was washed three times with water and with a 0.1 M HCl solution to reach a pH between 1 and 2. The organic phase was dried on sodium sulfate, filtered, and concentrated under reduced pressure. The residue was dispersed in a EtOH/H_2O mixture (20:20 (v/v)). A 0.3 M NaOH aqueous solution was added (4.25 mL) to reach a pH between 8 and 9. The resulting mixture was stirred at room temperature for 12 h. After centrifugation to remove nonsoluble impurities, the EtOH/H_2O mixture was evaporated. The residue was dissolved in water and then freeze-dried to give **18** as a white powder in 50% yield (480 mg).

^{1}H NMR (400 MHz, D_2O): δ 4.32 (dd, 1H, J = 5.55 Hz, C*H*−COO), 3.35 (m, 2H, $(CH_2)_{\alpha\ or\ \alpha'}$), 3.28 (m, 2H, $(CH_2)_{\alpha\ or\ \alpha'}$), 3.15 (m, 2H, $(CH_2)_{\gamma\ or\ \gamma'}$), 3.09 (m, 2H, $(CH_2)_{\gamma\ or\ \gamma'}$), 2.93 (dd, 2H, J = 4.19 Hz, CH_2−COO), 2.61 (m, 2H, $(CH_2)_{a\ and\ b}$−COO), 2.51 (m, 2H, CH_2−CO), 1.82−1.91 (8H, H (adamantane), $(CH_2)_{\beta\ or\ \beta'}$), 1.77 (m, 2H, $(CH_2)_{\beta\ or\ \beta'}$), 1.45−1.69 (28H, H (adamantane)).

^{13}C NMR (100 MHz, D_2O): δ 180.2, 179.9, 175.4, 175.3, 175.2 (6C, C═O), 54.3 (1C, (*C*H)−COO), 52.7, 51.9 (2C, *C*H_2 adamantane), 47.4, 45.4 (2C, $(CH_2)_{\alpha\ and\ \alpha'}$)), 43.9−43.6 (C adamantane), 38.2, 37.9 (2C, $(CH_2)_{\gamma\ and\ \gamma'}$), 37.7 (C adamantane), 33.7 (1C, (*C*H_2)-COO), 32.1, 29.3 (2C, $(CH_2)_{a\ and\ b}$−CO), 29.9, 29.2 (2C, $(CH_2)_{\beta\ and\ \beta'}$), 29,8 (C adamantane).

Table 2. Reaction Conditions for the Coupling of the Monomeric or Dimeric Species of CD and AD to HA−ADH

final polymer	dimethylacetal or aldehydic derivatives of CD or AD[b]		$NaCNBH_3$	yield (%)	deg of substitution[c]
HA−$(CD)_2$ **22**	**11**	0.15[a]	4.5[a]	88	0.05 ± 0.01
HA−CD **23**	**21**	0.3[a]	9[a]	77	0.05 ± 0.01
HA−$(AD)_2$ **26**	**13**	0.1[a]	4.5[a]	70	0.06 ± 0.01
HA−AD **27**	**25**	0.25[a]	7.5[a]	90	0.06 ± 0.01

[a] Number of molar equivalents with respect to the repeating disaccharide unit of HA−ADH. [b] Reactions were performed in water for the CD grafted HA samples and in a water/EtOH (3:2 (v/v)) mixture for the AD grafted HA samples. [c] Determined from ^{1}H NMR (D_2O, 80 °C).

ESI-HRMS: $[M + Na]^+$ *m/z* 721.41524 Calcd for $C_{38}H_{58}N_4O_8Na$; Found 721.4156. $[M-Na + 2Na]^+$ *m/z* 743.39718 Calcd for $C_{38}H_{57}N_4O_8Na_2$; Found 743.3983. $[M-2H + 3Na]^+$ *m/z* 765.37912 Calcd for $C_{38}H_{56}N_4O_8Na_3$; Found 765.3813.

Hyaluronan Derivatives. HA−ADH **19** was synthesized in our laboratory as reported in detail elsewhere.[15] The synthesis of the different host and guest polymers was based on a reductive amination-type reaction performed under reaction conditions similar to those used for the synthesis of HA−CD.[15] However, the solvent used for the preparation of the HA−AD and HA−$(AD)_2$ derivatives was a H_2O/EtOH (3:2 (v/v)) mixture instead of water. The procedure for the synthesis of HA−$(AD)_2$ is described below, as an example.

*HA−(AD)$_2$ **26.*** The first step consisted in deprotecting the aldehyde function of **13**. Thus, the acetalic dimer of adamantane **13** was dissolved in a CH_2Cl_2/TFA/H_2O (2:1:1 (v/v/v)) mixture (8 mL). The resulting mixture was stirred at room temperature for 90 min. Deprotection was followed by thin-layer chromatography using CH_2Cl_2/MeOH (9:1 (v/v)) as an eluant. The CH_2Cl_2/TFA/H_2O mixture was evaporated, and the product was dissolved three times in CH_2Cl_2 and concentrated to remove TFA. The resulting aldehyde **24** (0.25 mg, 0.4 mmol) dissolved in EtOH (26 mL) was added to HA-ADH **19** (1.64 g, 4 mmol) solubilized in a H_2O (400 mL)/EtOH (240 mL) mixture. The pH was adjusted at 5.1 using a 0.5 M NaOH solution. An aqueous solution (2 mL) of $NaCNBH_3$ (1.13 g, 18 mmol) was added. The resulting mixture was stirred at room temperature for 24 h. The pH of the reaction was then adjusted at 7.5 using a 0.1 M NaOH solution. After addition of NaCl at a concentration of 0.5 M, the modified HA was precipitated with EtOH in the 3/2 proportions for EtOH/H_2O. The precipitate was successively washed with different mixtures of EtOH/H_2O (7/3, 7.5/2.5, 4/1, 9/1) and then was filtered and dried (1.23 g, 2.74 mmol). The grafting degree of adamantane groups was found to be to 0.06 ± 0.01 by ^{1}H NMR.

^{1}H NMR (400 MHz, D_2O): δ 4.55 (H-1 from *N*-acetylglucosamine unit), 4.25 (H-1 from glucuronic acid), 3.9−3.1 (H-2, H-3, H-4, H-5, H-6 protons of HA), 2.32 (CH_2−CO), 2.48 (CH_2−CO), 2.2 (CH_2 from ADH), 2.05 (CH_2 from ADH), 1.85 (CH_3 from acetamide of HA), 1.3−1.55 (CH_2 from ADH, protons from adamantane).

Results and Discussion

1. Synthesis of the Dimers of AD and CD and Analysis of Their Binding Properties. We have described recently a new route to β-CD-conjugated HA which relies on the efficient selective functionalization of both HA and CD with reactive groups prior to their coupling.[15] The HA derivative was selectively modified with adipic dihydrazide (ADH) leading to HA−ADH and the β-cyclodextrin derivative was monofunctionalized by an aldehyde function on the primary face, allowing their coupling by a reductive amination-type reaction. We used a similar strategy for the covalent grafting of the dimeric CD and AD molecules on HA.

The strategy for the synthesis of the dimers of AD and CD having a protected aldehyde function was based on the preparation of a common intermediate **9** possessing primary amine groups for further attachment of the CD and AD molecules using a amine−acid coupling reaction (Schemes 1 and 2). However, since the AD dimer is not soluble in water which prevents investigation of complex formation by isothermal titration calorimetry, a water-soluble derivative possessing two carboxylate functions **18** was additionally synthesized (Scheme 3).

The common intermediate **9** was synthesized in four steps from bis(3-aminopropyl)amine **2**, commonly called norspermidine (Scheme 1). The primary amine functions of **2** were protected by benzyloxycarbonyl (CBz) groups using standard reaction conditions.[17] The low yield (30%) obtained is related to the concomitant formation of tri- and monofunctionalized compounds together with the difunctionalized derivative. Furthermore, the initial compound was also present in the mixture. Compound **4** isolated by silica gel column chromatography was then reacted with succinic anhydride **5** in the presence of pyridine, analogous to a previously reported procedure,[18] leading to the carboxylic acid derivative **6** with a 80% yield. Reaction of **6** with 2,2-dimethoxyethylamine **7** under peptide-like coupling conditions (dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), triethylamine (NEt_3)) gave diacetal **8** in a 51% yield. Deprotection of the primary amine functions of **8** was achieved by hydrogenolysis, leading to compound **9** with a 80% yield. The chemical structure of diamine **9** was confirmed by ^{1}H and ^{13}C NMR spectroscopy and electrospray mass spectrometry.

As mentioned above, cyclodextrin and adamantane having a carboxylic acid group were introduced on **9** under peptide-like coupling conditions (Scheme 2). Thus, the β-CD monocarboxylic acid derivative **10**, obtained according to our previously established methodology,[15] was coupled with diacetal **9** using *N*,*N*′-diisopropylcarbodiimide (DIC) and HOBt in DMF, resulting in the CD dimer **11** in a 67% yield. The latter was purified by precipitation in acetone allowing to remove the coupling reagents, followed by size exclusion chromatography to remove the diacetal derivative having only one CD grafted and nonreacted β-CD monocarboxylic acid.

Compounds **9** and (1-adamantyl)acetic acid (**12**) were coupled in DMF using DCC, HOBt, and Et_3N to provide the AD dimer **13** in a 43% yield. The latter was purified by silica gel column chromatography. The chemical purity and integrity of both dimers **11** and **13** were confirmed by ^{1}H NMR spectroscopy and electrospray mass spectrometry. Figure 3 diplays the ^{1}H NMR spectrum of dimer **11**. A splitting of the NMR signals, corresponding to the two branches A and A′, can be observed. The nonequivalence between the proton signals of the two branches likely results from the slow exchange rate at 25 °C between the two mesomeric forms related to the amide bonds. Thus, at room temperature, the molecule is locked under a conformation such as the single C−N bond achieves a double-bond character. Consequently, the two branches are under a different magnetic environment. This assumption was supported by the fact that the splitting of the proton signals, including the H-i and H-i′ ones, disappeared upon heating at 60 °C. The integration of characteristic signals, as for instance the β and β' CH_2 signals, in comparison with the β-CD H-1 proton signals provides evidence of the presence of two β-CD cavities per dimer moiety.

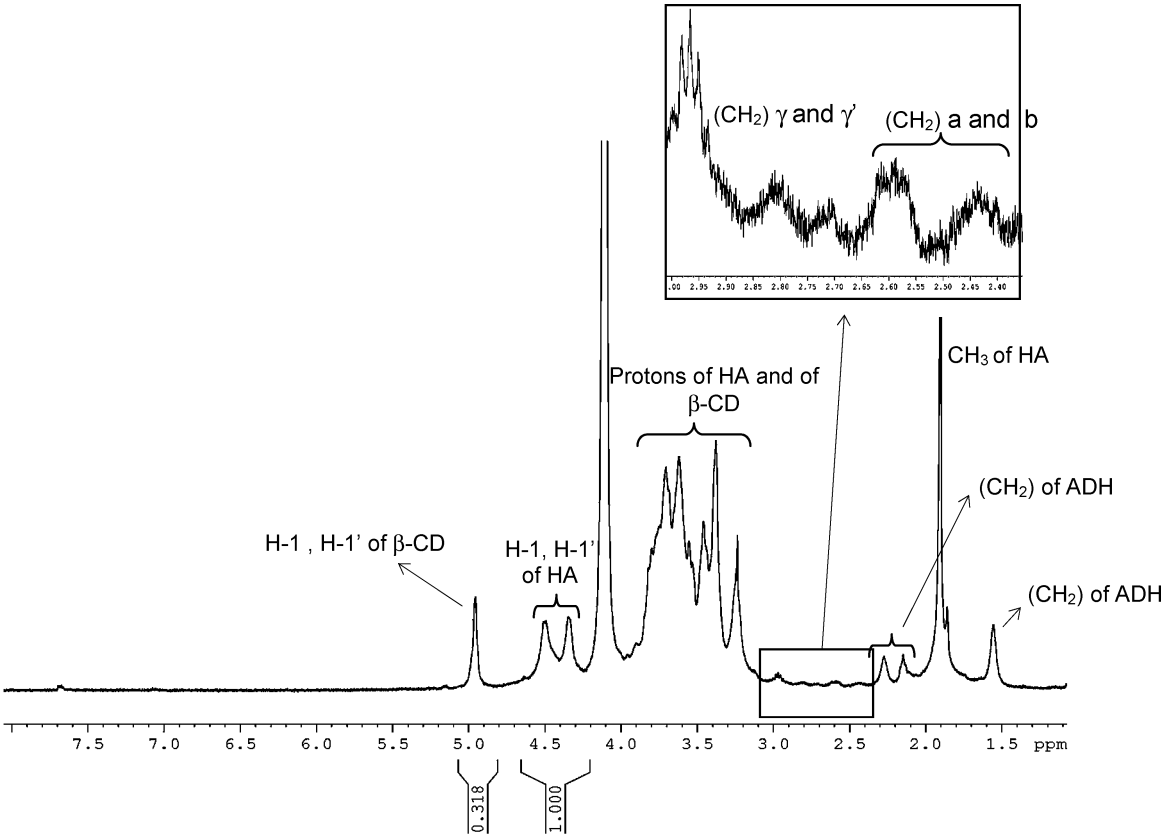


Figure 5. ^{1}H NMR spectrum (D_2O, 400 MHz, 80 °C, 6 mg/mL) of HA−$(CD)_2$ **22**.

The synthesis of the water-soluble AD dimer **18**, possessing two carboxylate functions, relies on a synthetic strategy similar to that described above for the preparation of the AD dimer **13**. Here, L-aspartic acid di-*tert*-butyl ester hydrochloride (**14**) was used instead of 2,2-dimethoxyethylamine (**7**) to be coupled with the carboxylic acid derivative **6** (Scheme 3). The *tert*-butyl esters were hydrolyzed in a CH_2Cl_2/TFA mixture.[19] The resulting carboxylic acid groups were then converted into carboxylates **18** by treatment with a 0.3 M NaOH aqueous solution in a EtOH/H_2O mixture.

Complex formation between the dimers of AD and CD was investigated by isothermal titration calorimetry. The data were compared with those resulting from the binding of sodium adamantane acetate (ADAc) by natural β-CD. As can be seen from Figure 4, the titration data for the AD dimer **18**/ CD dimer **11** system fitted perfectly the simplest model in which a single set of identical binding sites is present. The thermodynamic parameters derived from this model are given in Table 1. The stoichiometry found for the complexation between the dimers appears to be close to 1, indicating that one AD dimer is complexed by one CD dimer. The association constant K_a is 15 times higher than that found for the binding of ADAc by natural β-CD, and the enthalpy of binding is approximately double that for the β-CD/ADAc interaction. These results are in agreement with previously established results on the complexation properties of CD dimers and confirm the theory which predicts that the enthalpy variation for multivalent interactions is the addition of the corresponding monovalent interactions.[20,21] The unfavorable entropy found for the divalent interaction is probably due to a restriction of mobility and decrease of the degrees of freedom for the host and guest molecules. Thus, the obtained thermodynamic parameters provide evidence of the formation of divalent interactions involving the formation of two structurally similar inclusion β-CD/AD complexes per dimer molecule.

2. Synthesis of the Host and Guest Macromolecules and Analysis of Their Binding Properties. The dimeric CD and AD species were grafted on HA−ADH **19** using a reductive amination-type reaction under similar conditions to those recently described for the synthesis of HA(CD) (Scheme 4).[15] In order to study the influence of the energy and stability of the interchain junctions on the rheological properties of the supramolecular assemblies, monomeric derivatives of AD (adamantane acetaldehyde) and CD (β-CD derivative monofunctionalized by an aldehyde group on the primary face[15]) were also covalently introduced on HA−ADH using a reductive amination-type reaction to produce polymers having contents in CD and AD per chain similar to those of HA−$(CD)_2$ and HA−$(AD)_2$, respectively. The reaction conditions used for the synthesis of these host and guest HA derivatives are summarized in Table 2.

The coupling reaction between HA−ADH and CD dimer **20,** obtained after deprotection of the aldehyde group of dimer **11** by a mild acid treatment, was performed in water at an optimal pH value of 5.1, using sodium cyanoborohydride as a reducing agent. An excess of CD dimer was necessary because of side reactions (likely dimerization) involving the corresponding aldehyde **20**. As previously observed,[15] a pH increase of the mixture was observed since the beginning of the reaction, which could originate from the concomitant reduction of the C═N oxime bond of the β-CD cavity. Consequently, the pH was maintained by the dropwise addition of a 0.1 M HCl solution. The expected HA−$(CD)_2$ derivative **22** was isolated by a diafiltration process followed by freeze-drying. The chemical structure and integrity of HA−$(CD)_2$ were checked by ^{1}H NMR spectroscopy (see Figure 5). Digital integration of the NMR

Table 3. Influence of the Ionic Strength on the Thermodynamic Parameters for Inclusion Complex Formation of the Water-Soluble AD Dimer 18 with HA−$(CD)_2$ 22, Derived from Calorimetric Titration Experiments at 25 °C

[NaCl] (M)	[CD dimer] (mM)	[AD dimer] (mM)	$K_a \times 10^{-5}$ (M^{-1})	ΔH^0 (kJ/mol)	$T\Delta S^0$ (kJ/mol)	*n* (*n* AD dimer:1CD dimer)
0.025	0.0312	0.449	(0.69 ± 0.07)	−75.2 ± 4.1	−47.6 ± 0.02	1.03 ± 0.04
0.2	0.0302	0.448	(2.15 ± 0.11)	−45.5 ± 0.4	−15.08 ± 0.07	1.10 ± 0.10

Table 4. Thermodynamic Parameters for Inclusion Complex Formation of $HA(AD)_2$ 26 with the CD Dimer 11, Derived from Calorimetric Titration Experiments in NaCl (0.025 M) at 25 °C

[AD dimer] (mM)	[CD dimer] (mM)	$K_a \times 10^{-6}$ (M^{-1})	ΔH^0 (kJ/mol)	$T\Delta S^0$ (kJ/mol)	*n* (*n* CD dimer:1AD dimer)
0.0266	0.23	1.79 ± 0.10	−49.3 ± 0.4	−13.63 ± 0.09	0.59 ± 0.01

signals arising from the anomeric protons of HA and cyclodextrin gave a degree of substitution (DS) equal to 0.025 ± 0.005, which corresponds to a grafting degree of β-CD of 0.05 ± 0.01, as for HA−CD **23** (see Table 2). As mentioned above, a similar procedure was used for the synthesis of the HA−$(AD)_2$ **26** and HA-AD **27** derivatives. However, because of the very low water solubility of hydrophobic adamantane, the coupling reactions with HA−ADH were performed in a hydro-alcoholic solution (EtOH/H_2O). In the case of the synthesis of HA−$(AD)_2$, deprotection of the acetalic dimer of AD **24** before the coupling reaction was performed in a CH_2Cl_2/TFA/H_2O mixture. In addition, as observed for the synthesis of HA−$(CD)_2$, the coupling reaction with the AD dimer was not quantitative and an excess of aldehyde was necessary. Both HA−$(AD)_2$ and HA−AD derivatives were recovered by precipitation in a 0.5 M NaCl/ EtOH mixture followed by washing steps using water/ EtOH mixtures. The grafting degree of adamantane was determined by ^{1}H NMR spectroscopy and was found to be 0.06 ± 0.01 for HA−$(AD)_2$ and HA−AD.

The inclusion ability of the grafted dimers of AD and CD was then investigated by ITC experiments, using CD dimer **11** and the water-soluble dimer of AD **18** as model guests, respectively. In the case of the complexation between the HA−$(CD)_2$ derivative and the water-soluble AD dimer, ITC experiments were performed by varying the ionic strength as the presence of electrostatic interactions between negatively charged AD dimer and HA−$(CD)_2$ may influence the complexation thermodynamics. Table 3 gives the thermodynamic parameters obtained for the complexes between $HA(CD)_2$ and the free AD dimer in 0.025 and 0.2 M NaCl. A profound effect of electrostatic interactions on the complexation between the AD dimer **18** and HA-$(CD)_2$ **22** is revealed from the data of Table 3. The enthalpy and entropy values tend to increase when the concentration of NaCl is increased, as already observed for the complexation of ADAc by HA(CD).[15] This resulted in a higher value of the association constant in 0.2 M NaCl, where electrostatic repulsions can be considered to be completely screened. Nevertheless, the association constant found with the grafted CD dimer is about 5 times lower than that with the free one, which is due to an unfavorable entropic contribution as a result of the grafting on the polymer chain. It should be noted that a decrease of the association constant was also found in the case of HA−CD.[15] Concerning the complexation between $HA(AD)_2$ **26** and CD dimer **11**, the thermodynamic parameters given in Table 4 reflect a divalent interaction with a very high association constant. The enthalpy and entropy values are close to those obtained for the AD dimer **18**/HA−$(CD)_2$ **22** complex. The value of the stoichiometry *n* of 0.59 instead of the expected value of 1 suggests that one part of AD molecules (41%) is not available for the complexation. The value lower than one is reflected on the thermogram (see Figure 6). Indeed, the equivalence point of the titration appears for a molar ratio of ~0.5 instead of ~1, as can be seen on the thermogram of Figure 4. This result might be related to the existence of intra- or/and interchain hydrophobic interactions between the grafted AD moieties. Given the rather high AD/β-CD affinity, one would have expected that the AD/AD interactions would have been much weaker and, consequently, could be dissociated by the addition of the CD dimers. Nevertheless, viscosimetry experiments demonstrated the impossibility to completely remove AD−AD interactions by the addition of excess free β-CD (data not shown). Indeed, even in the presence of 3 mol equiv of β-CD with respect to the grafted AD, the viscosity of solutions of HA(AD) was shown to be higher than that of solutions of initial HA. It should be noted that hydrophobic associations likely occur between the side chains containing the two adamantanes. ITC experiments revealed a 1:2 stoichiometry of binding for the AD dimer **18**/β-CD system (data not shown), suggesting that all the AD groups are available to interact with β-CD. Consequently, this rather precludes hydrophobic interactions between adamantanes within the side chains.

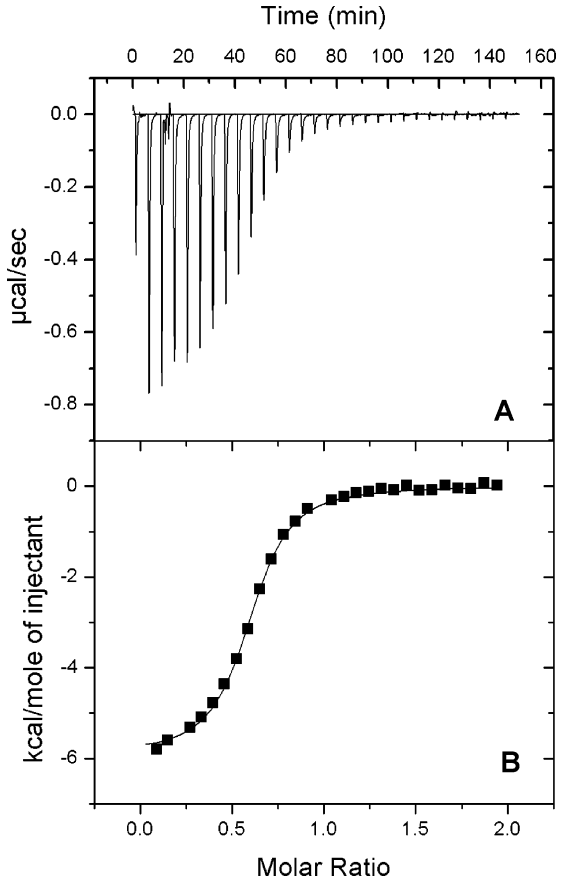


Figure 6. Calorimetric titration of $HA(AD)_2$ **26** with the CD dimer **11** in 0.025 NaCl at 25 °C (see Table 4). (A) Raw data for 30 sequential injections (10 μL per injection) of CD dimer ([CD dimer] = 0.230 mM) injected into $HA(AD)_2$ solution ([AD dimer] = 0.0266 mM). (B) The integrated curve showing experimental points and the best fit for the titration of $HA(AD)_2$ with the CD dimer.

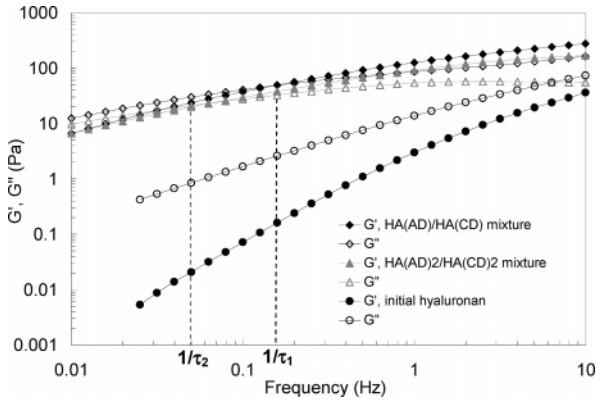


Figure 7. Comparison of the storage and loss moduli as a function of frequency for a HA(AD) (DS = 0.06)/HA(CD) (DS = 0.05) mixture (C_p = 10 g/L), a HA(AD)$_2$ (DS = 0.03)/HA(CD)$_2$ (DS = 0.025) mixture (C_p = 10 g/L), and a hyaluronic acid solution (C_p = 30 g/L). Solvent: 0.025 M NaCl, 25 °C.

3. Formation of Supramolecular Assemblies in Aqueous Solution. The supramolecular assemblies were prepared by mixing aqueous solutions of HA−CD and HA−AD, on the one hand, and of HA−(CD)$_2$ and HA(AD)$_2$, on the other. These mixtures lead in 0.025 M NaCl to the formation of macroscopically transparent "gels" for total polymer concentrations 1.5 times higher than the critical overlap concentration C^* (~1.3 g/L) of initial HA. The formation of such networks results from the simultaneous formation of many complexes between the monomeric or dimeric CD and AD molecules grafted along the HA chain. It should be noted that 0.025 M NaCl was selected as the salt concentration for the formation of the assemblies in aqueous medium, since NaCl concentrations higher than 0.05 M resulted in a phase separation phenomenon, whereas no gel formation was macroscopically observed in pure water.

Figure 7 compares the frequency dependence of the dynamic rheological moduli, G' and G'', of the HA(AD)/HA(CD) (total polymer concentration C_p = 10 g/L) and HA(AD)$_2$/HA(CD)$_2$ (C_p = 10 g/L) mixtures with that of a solution of initial HA (C_p = 30 g/L). As already mentioned, the HA−AD and HA−(AD)$_2$ derivatives, on the one hand, and HA−CD and HA−(CD)$_2$ derivatives, on the other, possess the same average number of AD molecules and CD cavities per HA chain, respectively. From Figure 7, it can be observed that the values of the storage and loss moduli for the mixtures of modified HA samples are much higher than those obtained for the solution of initial HA, although the concentration of the latter is 3 times higher than that of modified polymers in the mixtures. Furthermore, G' is larger than G'' within a large range of frequency for the HA(AD)/HA(CD) and HA(AD)$_2$/HA(CD)$_2$ mixtures, reflecting a viscoelastic behavior, contrary to the solution of initial HA which exhibits a viscous character within the whole range of frequencies covered. The viscoelastic properties of the mixtures look like those of very high molecular weight and/or concentrated polymer solutions as observed in the case of modified chitosan.[1] As no maximum for the loss modulus can be observed, the intersection of the G' and G'' curves was considered in first approximation as the inverse of the characteristic relaxation time of the mixture.[22] If we compare now the frequency dependence of the G' and G'' moduli for the HA(AD)/HA(CD) and HA(AD)$_2$/HA(CD)$_2$ mixtures, we can observe interesting differences related to the nature of the interchain junctions. The characteristic relaxation time (τ_2) is longer whereas the values of the G' and G'' moduli are lower for the HA(AD)$_2$/HA(CD)$_2$ system. Since this system is stabilized by pairs of complexes resulting in a higher binding constant compared to that of the single CD/AD complex as demonstrated by ITC, the HA chains, on which divalent complexes play the role of sticky points, will require a longer time for relaxation. Moreover, the lower values found for the G' and G'' moduli may reflect the smaller density of effective interchain junction as the content in AD and CD is similar for both mixtures, but they are distributed by pairs along the polymer chain in the case of the "bi-sticker" system. Given the exceptional increase of the association constant found by ITC for the complex between the free dimers of CD and AD, we could have expected a much slower dynamics for the system with bi-stickers than that for the corresponding mono-sticker system. Gain resulting from divalent interactions is limited probably due to the polymer chain effect which involves steric restrictions for complex formation and by the presence of hydrophobic AD/AD interactions, which compete with the formation of inclusion complexes between the grafted dimers. These points will be discussed in a future paper.

In conclusion, novel hyaluronic acid-based supramolecular assemblies stabilized by pairs of CD/AD complexes, which show distinct viscoelastic properties in aqueous solution compared to those of assemblies resulting from single CD/AD complexes, were obtained. Their preparation involved the controlled synthesis of suitable dimeric CD and AD molecules in order to be selectively grafted on a HA derivative possessing pendent reactive hydrazide groups along the chain.

Investigation of the complexation properties of the free and grafted AD and CD dimers by isothermal titration calorimetry experiments demonstrated a marked increase of the association constant compared to the single CD/AD complex, which could be attributed to divalency. Nevertheless, in the case of the host and guest macromolecules, the inclusion abilities appear to be affected by several factors, such as electrostatic repulsions between the host and guest (macro)molecules, restrictions of mobility due to the fixation of the host or guest molecule on the polymer chain, and competitive hydrophobic AD/AD interactions (in the case of the HA(AD)$_2$ derivative). These factors, acting with a more or less extent, lead to lower binding constants compared to the free dimers and/or the unavailability of one part of AD molecules for complexation.

Dynamic rheological measurements performed on the HA(AD)/HA(CD) and HA(AD)$_2$/HA(CD)$_2$ mixtures demonstrated a viscoelastic behavior for these assemblies contrary to the solution of initial HA. In addition, the dynamics for the bi-sticker system was shown to be slower than that for the corresponding mono-sticker system, although the slowing down was not as large as expected from the values of the binding constant for the free divalent CD/AD complex. These supramolecular assemblies represent a new class of temporary networks stabilized by well-defined junction points and constitute a relevant model involving punctual multivalent interactions. Additional rheological experiments were performed by varying different physical and chemical parameters in order to get information about the mechanism of formation of these assemblies. They will be presented in a future paper.

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