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Effect of acetylation on physico-chemical properties of bacterial and algal alginates in physiological sodium chloride solutions investigated with light scattering techniques

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Abstract

Static light scattering measurements were performed to investigate the effect of acetylation on the physico-chemical properties of alginates in salt solutions from different sources. Although chemical acetylation reduces the molar mass of algal alginate by 27%, it has a size increasing effect on the alginate clusters. The influence of molar mass on the dimensions of polymer aggregates is outweighed by the effect of acetyl groups. Negative virial coefficients are found which become more positive for acetylated alginates. They give another explanation of the size increasing effect of acetylation on the cluster size since acetylated alginates show a better interaction mainly with the water molecules of the salty solution than unacetylated alginates do.

Keywords: Alginate; Acetylation; Light scattering; Characterization

1. Introduction

Alginates are copolymers of β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G), both linked together by 1–4 linkages. They can be isolated from different natural sources. Commercially available alginates are used in a variety of technical applications like thickening and gelling agents in the food industry or to immobilize cells in the biotechnological industries (Onsøyen, 1996; Rehm & Valla, 1997). They are currently extracted from brown seaweeds which are harvested throughout the year.

Alginate like polymers are also produced by several bacteria of the *Pseudomonas* and *Azotobacter* genera (Rehm & Winkler, 1996). Both types of alginates vary in several ways. Although algal alginates are available in a big variety of molar masses, bacterial alginates generally have a higher degree of polymerization than algal alginates, yielding higher molar masses as demonstrated by us before (Windhues & Borchard, 2002b). Furthermore, both types of alginates can be differentiated by their distribution of monomer sequences. While algal alginates show alternating sequences of M and G as well as homopolymeric monomer units (Schürcks, Wingender, Flemming, & Mayer, 2002), the oligomeric guluronic sequences are missing in bacterial alginates (Skjåk-Bræk, Grasdalen, & Larsen, 1986). The lack of homoguluronic units may be explained by the partial acetylation on O-2 and O-3 position of the mannuronate units of bacterial alginates which is missing for algal alginates. Acetyl groups protect the M-residues from conversion to G-residues by C-5-epimerase and give bacteria a way to control the alginate composition and properties (Skjåk-Bræk, Larsen, & Grasdalen, 1985). Hence, acetylation can be seen as the biggest difference of both alginates from a chemical point of view.

Since bacterial alginates become more and more important for industrial applications and algal alginates often serve as model substances for bacterial alginates, it is important to know the effect of acetylation on the physico-chemical properties of different alginates. The approach of this study was to compare algal alginate and chemically acetylated algal alginate to bacterial alginates of two different *Pseudomonas aeruginosa* strains. The strain FRD1 is mucoid and was isolated from the sputum of a patient with cystic fibrosis. Its alginate has an acetyl group content of 8.1% (Tielen, 1999). The strain FRD1153 is derived from FRD1 as a mutant in *algJ3*-gen and has a defect of O-acetylation (Franklin & Ohman, 1993).

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The amount of acetyl groups is reduced to 0.7% (Tielen, 1999). As a method for their physico-chemical characterization light scattering techniques were used. They give information about the molar mass of the polymers, their size and shape in highly diluted solution and the second virial coefficients as a measure of the preferred particle interactions in solution.

2. Materials and methods

The algal alginate Manucol DM, Lot No. 770111, sample Ref. 400066486, was kindly given to us by Monsanto, Hamburg, Germany. As to manufacturers specifications, it has a mannuronate content of 65–70% and a guluronate content of 30–35%. Prior to its use it was dialyzed against water and freeze dried for storage.

Acetylation was done according to a slightly modified procedure described by Skjåk-Bræk (Skjåk-Bræk, Paoletti, & Gianferrara, 1988). While stirring 1 l calcium chloride solution (0.1 mol/l), 500 ml of an alginate solution (2 mg/ml) were dripped into it. After decantation of the supernatant fluid, 500 ml pyridine were added to the precipitate before storage for 1 day at room temperature. The pyridine was decanted and the alginate beads were covered with 500 ml of a mixture of 50% pyridine and 50% acetic anhydride. This reaction mixture was stored at room temperature for 2.5 h shaking it by hand every 10 min. The excess pyridine/acetic anhydride mixture was then decanted and the alginate beads were washed three times with 250 ml acetone and three times with 250 ml deionized water before dissolving them in 250 ml Na_2EDTA (0.05 mol/l). After dialyzation for 24 h against 5 l sodium chloride solution (0.1 mol/l) and deionized water, the alginate was freeze dried for storage. The acetyl content was checked by a photometric assay according to Hestrin (Hestrin, 1949). With this method, the alginate prepared above was found to have an acetyl content of 9.3% (w/w).

Bacterial alginates were extracted from agar grown biofilms as described by Wingender et al. (Wingender, Strathmann, Rode, Leis, & Flemming, 2001). Petri dishes with 25 ml Pseudomonas Isolation Agar (PIA from Difco) enriched with 5% (v/v) gylcerol were used as growth media. The inoculum consisted of a bacteria suspension prepared from bacteria cultures pre-grown overnight at 36 °C. These cultures were suspended in 5 ml of sterile sodium chloride solution (8.2 g/l) to a cell density of approx. 10^8 cells/ml. 0.1 ml of this suspension was plated on PIA and incubated for 24 h at 36 °C. Separation of the EPS from the cells was done after scraping the biofilm off the agar with a spatula and suspending it in sodium chloride solution (8.2 g/l). The cells were dispersed by stirring the suspension with a magnetic stirrer for 30 min before they were separated from the EPS by centrifugation at 40,000 g for 2 h at 10 °C. The alginate was extracted from the supernatant solution as a precipitate collected on a glass sintered filter after addition of a threefold volume of ice-cold ethanol (95% v/v) and storage in an ice bath for 30 min. This precipitate was washed twice with ice-cold ethanol (95% v/v) and once with ice-cold absolute ethanol before it was dried over phosphoruspentoxide under a vacuum. Final purification of the precipitate was done by dissolving it in 50 mM Tris–HCl buffer with 10 mM magnesium chloride at a concentration of 2.5 mg/ml. This solution was incubated for 4 h at 36 °C in presence of DNase I (10 μg/ml) and RNase A (10 μg/ml) before it was stored with addition of 12.5 μg/ml Pronase E overnight at 36 °C. Centrifugation at 40,000 g for 30 min, twofold dialyzation against two proportions of 5 l deionized water and reprecipitation with ethanol gave the alginate, that was freeze dried for storage after dissolving it in deionized water.

The samples for light scattering were prepared as described in our previous papers (Windhues & Borchard, 2002a,b). Sodium chloride solution (8.2 g/l) was used as a solvent for the concentration series to take into account the polyelectrolyte character of alginate. A solution with a concentration of 0.5 g/l was stirred overnight and then heated to 45 °C to completely dissolve the polymers. This stock solution was diluted to achieve polymer concentrations of 0.4, 0.3, 0.2 and 0.1% by weight. These solutions were centrifuged overnight at 3000 g to free them from dust. As already mentioned (Windhues & Borchard, 2002b), no sedimentation of the polymers is to be expected under these conditions.

The static light scattering experiments were carried out at angles from 30° to 150° in steps of 5° with an ALV 3000 light scattering equipment of ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany. The incident light was provided by a frequency doubled Nd/YAG laser with a wavelength of 531 nm (ADLAS, Lübeck, Germany). Heating and cooling of the probes was started 30 min before the measurements were performed in a bath of toluene with adjusted temperature. The refractive index increments of the solutions were measured with the interferometric refractometer ScanRef (NFT Nanofilm Technologie GmbH, Göttingen, Germany).

3. Results

The light scattering results are obtained from Eq. 1 as derived by Zimm (Zimm, 1948)

$$\frac{k\rho_2}{\Delta R_\Theta} = \left[1 + \frac{16}{3}\frac{\langle r_g^2\rangle}{\lambda_0^2}\sin^2\frac{\Theta}{2}\right]\left[\frac{1}{M_w} + 2A_2\rho_2\right] \qquad (1)$$

with

$$\Delta R_\Theta = R_{\Theta,m} - R_{\Theta,s}; \qquad k = \frac{4\pi n^2}{N_A\lambda_0^2}\left(\frac{\partial n}{\partial \rho_2}\right)^2_{P,T,\lambda_0}$$

$R_{\Theta,m}$: Rayleigh ratio of the mixture; $R_{\Theta,s}$: Rayleigh ratio of the solvent; $\langle r_g^2\rangle$: mean squared radius of gyration

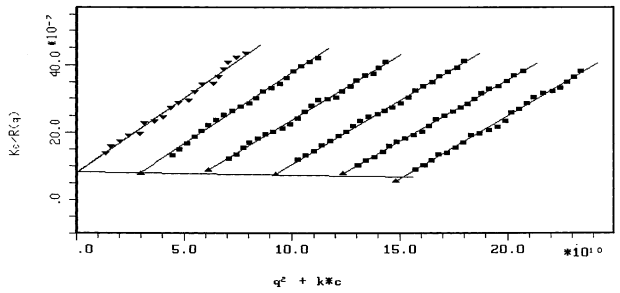

Fig. 1. Zimm plot of light scattering data of alginate solutions of *P. aeruginosa* FRD1 at 35 °C.

(z-average); n_1 : refractive index of the solvent; Θ: scattering angle; A_2: second virial coefficient; ρ_2: concentration of the polymer; M_w: molar mass (weight average); N_A: Avogadro constant; λ_0: wave length of incident light; $(\partial n/\partial \rho_2)_{P,T,\lambda_0}$: refractive index increment.

Extrapolation to zero scattering angle and zero concentration yield straight lines whose y-intercepts reflect the reciprocal molar mass of the polymer. The second virial coefficient of the system is obtained as the slope of the first extrapolation whilst the slope of the line obtained from the second extrapolation reflects the radius of gyration of the dissolved polymer. With this method, standard deviations of the calculated parameters are typically in the range of 5%. From comparison of the measured scattering functions to scattering functions calculated for certain molecule shapes, the shapes of the particles can be determined.

The Zimm plot of alginate from *P. aeruginosa* FRD1 at 35 °C is shown in Fig. 1 as an example of the shape of the Zimm plots obtained from light scattering data of the other investigated systems.

The straight lines of the Zimm plots fit the theoretically derived relation of angle and concentration dependant intensity of the scattered light for highly diluted polymer solutions. Hence a calculation of the molar mass, radius of gyration and second virial coefficient according to Eq. (1) is reasonable.

Fig. 2 is a plot of the scattering function of alginate produced by the *P. aeruginosa* strain FRD1. This plot obtained with the light scattering data of the aqueous alginate solutions at 35 °C is exemplary shown for

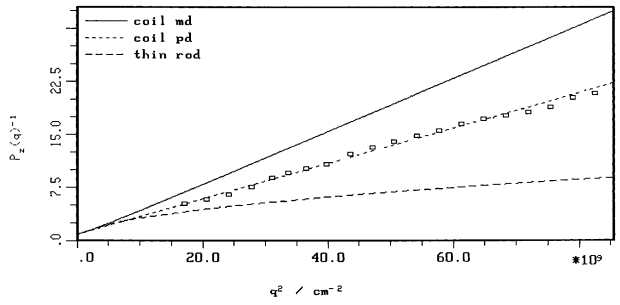

Fig. 2. Scattering function of alginate solutions of *P. aeruginosa* FRD1 at 35 °C (coil md: monodisperse coil; coil pd: polydisperse coil; thin rod: rod-like structure).

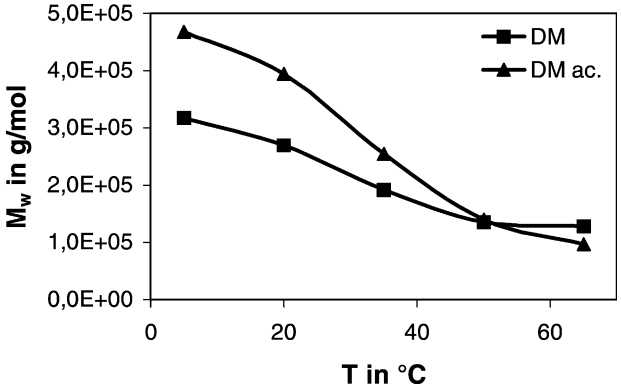

Fig. 3. Temperature dependence of the molar mass of algal alginates in physiological salt solutions.

the scattering functions of the other systems which have similar shapes.

It can be easily seen that the measured scattering function corresponds to the calculated one for polysdisperse coils. Hence all alginate macromolecules are coil shaped in the investigated temperature range due to their similar scattering functions.

Figs. 3 and 4 show the weight averaged molar masses of the investigated alginates at various temperatures.

All alginates show a linear decrease of their molar masses with rising temperatures. The molar masses of algal alginates vary from 317,000 g/mol at 5 °C to 128,000 g/mol at 65 °C for the alginate Manucol DM and 468,000 g/mol at 5 °C to 97,000 g/mol at 65 °C for acetylated alginate. Bacterial alginates have molar masses ranging from 953,000 g/mol at the lowest investigation temperature to 734,000 g/mol at 65 °C for unacetylated alginate from FRD1153 and 1,640,000 g/mol at 5 °C to 990,000 g/mol at 65 °C for alginate extracted from *P. aeruginosa* FRD1.

The radii of gyration show similar trends as shown in Figs. 5 and 6.

Manucol has mean squared radii ranging from 1.61×10^{-10} cm^2 at 5 °C to 6.84×10^{-11} cm^2 at the highest temperature while the mean squared radii of gyration are running from 2.41×10^{-10} cm^2 at 5 °C to 9.01×10^{-11} cm^2

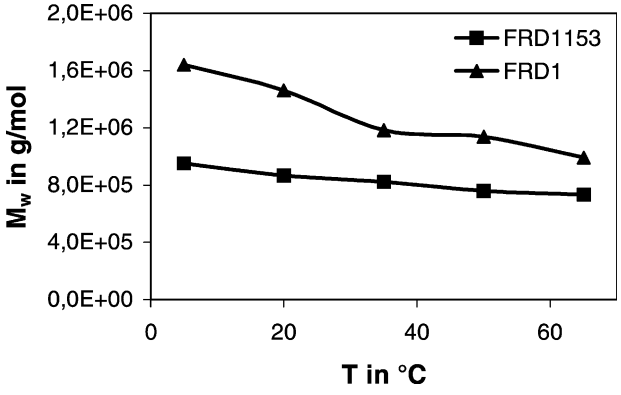

Fig. 4. Molar mass of bacterial alginates at different temperatures in aqueous sodium chloride solutions.

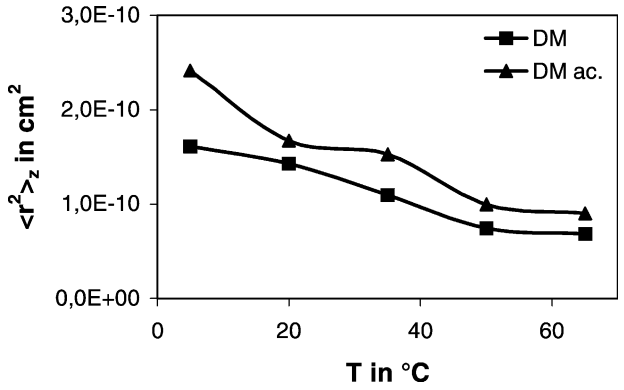

Fig. 5. Radii of gyration of highly diluted algal alginates.

at 65 °C after acetylation. The dimensions of alginate coils extracted from biofilms of *P. aeruginosa* FRD1153 are in the range of 6.93×10^{-10} cm^2 at 5 °C to 3.58×10^{-10} cm^2 at 65 °C. The strain FRD 1 produces alginate with molecule sizes varying from 8.44×10^{-10} cm^2 at 5 °C to 2.91×10^{-10} cm^2 at 65 °C.

Second virial coefficients of the investigated solutions are small and negative for all four systems as can be seen in Figs. 7 and 8.

They increase with rising temperatures and range from -1.18×10^{-3} mol cm^3/g^2 at 5 °C to -2.42×10^{-4} mol cm^3/g^2 at 65 °C for Manucol. Solutions of acetylated Manucol have virial coefficients varying from -6.13×10^{-4} mol cm^3/g^2 at 5 °C to -3.32×10^{-4} mol cm^3/g^2 at 65 °C. Solutions prepared from alginates of FRD1153 have virial coefficients running from -8.20×10^{-3} mol cm^3/g^2 at 5 °C to -5.99×10^{-3} mol cm^3/g^2 at 65 °C while solutions of alginate from strain FRD1 have second virial coefficients from -5.19×10^{-3} mol cm^3/g^2 at the lowest temperature to -3.40×10^{-3} mol cm^3/g^2 at 65 °C.

4. Discussion

The molar masses of all alginates are temperature-dependent and show a decrease with rising temperature.

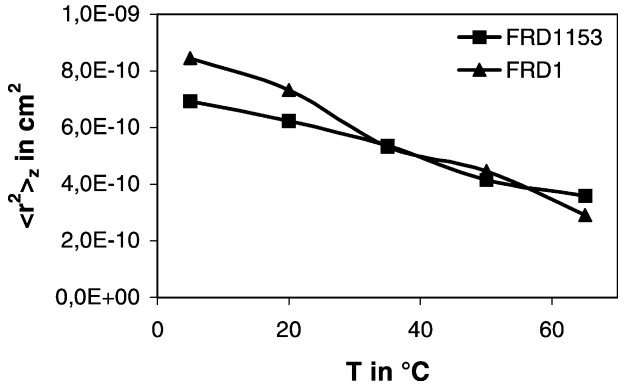

Fig. 6. Radii of gyration of bacterial alginates vs. temperature in 0.15 m salt solutions.

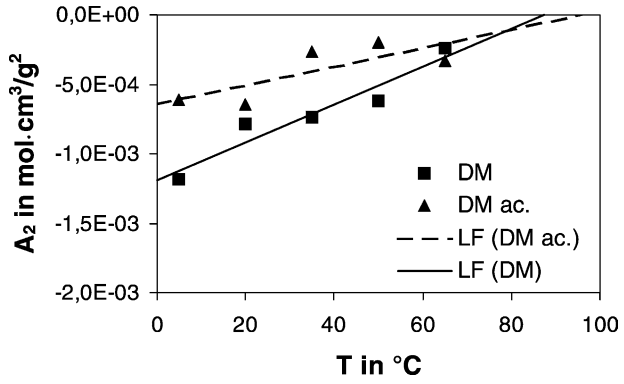

Fig. 7. Temperature dependence of the second virial coefficients of diluted algal alginates.

This decrease is an indication of association phenomena of the polymers which become less strong when heating the solutions. Hence the true weight average molar masses of the polymers cannot be determined. Since higher investigation temperatures may affect the thermal stability of the biopolymers, the values calculated for the highest temperature seem to be closest to the true values of the unassociated molecules. Figs. 3 and 4 show that the molar masses of the polymers seem to be independent of temperature above 60 °C.

Applying this thought to the molar mass of algal alginate, the chemical acetylation process causes a slight break down of the macromolecules. At 65 °C unacetylated alginate has a molar mass of 128,000 g/mol corresponding to a degree of polymerization of 730. With a degree of acetylation of 9.3%, an increase of the molar mass of 4% after acetylation is expected. Its molar mass is reduced to 97,100 g/mol though. The degree of polymerization can be calculated to be 27% lower than the degree of polymerization of the initial alginate. Interestingly the molar mass of acetylated Manucol shows a stronger increase when cooling than the initial alginate does. Obviously, acetyl groups support the association process. They may act as hooks or small side chains which support the formation of polymer aggregates.

For bacterial alginates the presence of acetyl groups has a different effect. Acetylated alginate from FRD1 has a molar

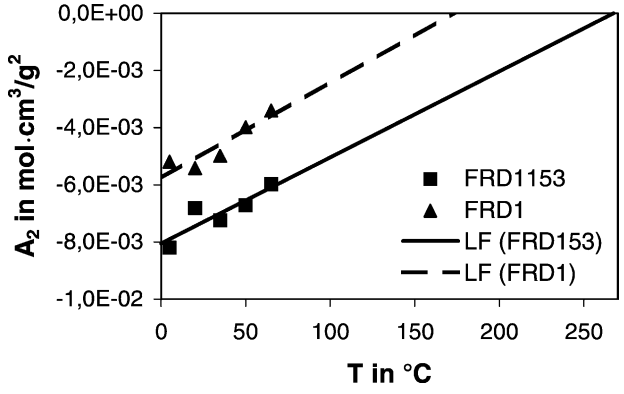

Fig. 8. Second virial coefficients of alginate solutions at different temperatures.

mass of 990,000 g/mol at 65 °C which corresponds to a degree of polymerization of 5400. This is 26% higher than the degree of polymerization of alginate from FRD1153 which is 4200. A possible explanation of this effect is a protection of acetylated alginate against alginate lyasis that breaks up the alginate when it is secreted from the cells. In correspondence to algal alginates, the polysaccharide produced by FRD1 shows a stronger increase of its molar mass with cooling than alginate from FRD1153 does which can be explained by the same effect mentioned before.

Since the alginate molecules are coil shaped over the whole temperature range, no effects on the mean squared radii of gyration caused by shape transitions are expected. The radii of gyration of algal alginates show characteristic temperature dependencies, as well. Over the whole temperature range clusters from chemically treated alginate are bigger than aggregates from unacetylated alginate. This again may be explained by acetyl groups acting as small side chains stiffening the polymer strands and widening the clusters. Aggregates of the acetylated alginate with a smaller molar mass are bigger in size than those of the unacetylated alginate. Obviously, size effects correlated to the molar mass are outweighed by size increases caused by the presence of acetyl groups. Theoretical consideration yielded size increases of 20% after acetylation (Stokke & Smidsrod, 1993) due to a restriction of the conformational freedom of the polymer chains probably due to a hindered rotation of the monomers. From viscosity investigations published elsewhere size increases were found for degrees of acetylation of up to 11% (Skjåk-Bræk, 1998). Both results can be verified. The size increases of the clusters when cooling the probes support the result of stronger association at lower temperatures mentioned before. Similar results are found for bacterial alginates. The clusters of alginate produced by FRD1153 are in general smaller in size than the clusters of the acetylated alginate extracted from FRD1 biofilms. These results correspond to those found for algal alginates.

The second virial coefficients of all systems are negative. Hence the polymers interact preferably with themselves rather than interacting with the solvent molecules of the highly diluted solution. The trend of stronger particle interactions at lower temperatures can be verified by the virial coefficients, as well. In general they become more negative when cooling the solutions. Virial coefficients of solutions of Manucol are more negative than the virial coefficients of solutions of acetylated algal alginate. One possible explanation is the difference of their molar masses since polymers with a higher degree of polymerization tend to have smaller virial coefficients (Fujita, 1990). Additionally the introduction of acetyl groups may ease the alginate–solvent interaction supporting the association of algal alginates. A side effect of the better water–alginate interaction may be a widening effect on the clusters in solution yielding bigger aggregates. For bacterial alginates, this effect most likely outweighs the influence of the difference in molar masses. Although alginate from FRD1 has a higher molar mass than alginate from FRD1153, the amounts of the negative virial coefficients of their solutions are smaller. Extrapolation of the virial coefficients to zero yield the so called Θ-temperature of the system where no particle interactions are preferred. They can be calculated to be 87 °C for Manucol, 96 °C for acetylated alginate, 267 °C for alginate produced by strain FRD1153 and 173 °C for the exopolysaccharide extracted from biofilms of FRD1. The regression coefficients are between 0.6 and 0.9. As mentioned by us elsewhere, the results of these extrapolations are not exact enough to state anything about the relation of Θ-temperature and degree of acetylation (Windhues & Borchard, 2002b).

5. Conclusions

The effect of acetylation on the physico-chemical properties of algal and bacterial alginates could be investigated with static light scattering measurements. Chemical acetylation of algal alginate reduces its degree of polymerization by 27%. The defect of O-acetylation of alginate produced by *P. aeruginosa* FRD1153 yields a decrease of its degree of polymerization of 26% compared to alginate extracted from biofilms of *P. aeruginosa* FRD1 due to the loss of protection against alginate lyasis. Furthermore acetyl groups have a widening effect on the alginate clusters that outweighs size effects correlated to different molar masses. The second virial coefficients are slightly negative for all systems. For bacterial and algal alginates, acetyl groups ease the interaction of the macromolecules with the solvent molecules which raises the virial coefficients and widens the clusters.

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