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Self-Assembly of a β-Sheet Protein Governed by Relief of Electrostatic Repulsion Relative to van der Waals Attraction

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Using a synthetic oligopeptide, n-FKFEFKFEFKFE-c (KFE12), representative of a class of peptides that can undergo self-assembly into a three-dimensional matrix biomaterial, we show that the self-assembly occurs when solution conditions reduce intermolecular electrical double-layer repulsion below van der Waals attraction in accord with DLVO theory. This theory predicts that a critical coagulation concentration of counterions should be required to allow assembly and that this concentration should be inversely proportional to the valence of the counterion raised to the sixth power. Our experimental results show that KFE12, at low pH, exhibits critical coagulation concentrations in each of three different salt solutions, KCl, K_2SO_4, and $K_3Fe(CN)_6$, and that the relative values of these critical concentrations follow the predicted dependence upon anion valence. The theory further predicts that self-assembly should occur when the oligopeptide is electrically neutral even in the absence of exogenous salt. Our experimental results show that KFE12 indeed forms gels when neutralized with NaOH. Thus, we have gained fundamental theoretical understanding of how to control the assembly of this class of oligopeptide-based biomaterials.

Introduction

An especially desirable feature of a biomaterial useful for applications in drug delivery, wound healing, and tissue engineering is the capability for self-assembly into a three-dimensional matrix scaffold in situ under physiological conditions.[1] The class of oligopeptides originally discovered by Zhang et al.[2] exhibits this property, along with others such as favorable cell interactions.[3,4] However, to develop reliable technologies based on this class of biomolecules, it is essential to understand the fundamental principles that govern self-assembly.

The particular oligopeptide, named KFE12 (shown in Figure 1), is a derivative of a sequence originally discovered by Zhang et al.[2] in the protein Zuotin and has alternating hydrophobic side chains, phenylalanine, and charged side chains, lysine and glutamic acid. Upon addition of a sufficient concentration of salt, a network of filaments forms which behaves as an elastic gel.[5] Our aim is to produce a material that would undergo a transition from being soluble (forming a viscous solution) outside of the human body to a gel once it is injected into the human body. Armed with theoretical understanding concerning fundamental self-assembly principles, we should be able to alter the underlying amino acid sequence to obtain a material suitable for specific application—not only in terms of self-assembly conditions but also in terms of mechanical and cell-interaction properties. In addition, gaining this theoretical understanding for this oligopeptide system will give us insight into the driving forces for assembly of more complex proteins such as β-amyloid and prion proteins. If we are able to understand why these molecules assemble, we may be able to predict conditions that will prevent plaque formation.[6]

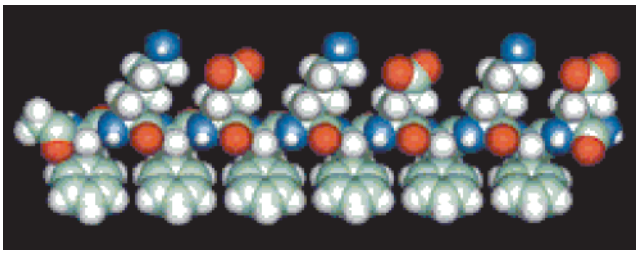

Figure 1. Schematic molecular model of FKFEFKFEFKFE (KFE12). Carbon atoms are pale green, Oxygen atoms are red, Nitrogen atoms are blue, and Hydrogen atoms are white. In this conformation (antiparallel β-sheet), all of the hydrophobic phenylalanine side chains face in one direction, and all of the lysine and glutamic acid side chains face in the other direction. On the polar face, glutamic acids alternate with lysines. Previous circular dichroism studies have shown that EAK family oligopeptides form β-sheets[2].

A wealth of information exists on the conditions under which β-sheet proteins assemble,[7,8] the kinetics of fibril formation,[9,10] and the resultant morphology of the fibrils.[11,12] Current understanding of the driving forces for self-assembly, however, is limited to the qualitative concept that the proteins assemble in water due to the hydrophobic effect[13] but that charged and polar side chains also play important roles in

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determining what conditions support assembly and what conformation the aggregate will adopt.[14,15] Furthermore, it is unclear whether juxtaposition of unlike charges increases stability of the preferred confomer or juxtaposition of like charges decreases stability of all other possible confomers.[16−20]

We hypothesize that KFE12 assembles because of the hydrophobic effect (the entropy of water is greater when not in contact with a hydrophobic side chain) but that assembly will be hindered by like-charge repulsion from the charged faces. Hence, the oligopeptides will remain unassembled when like-charge repulsion dominates over the hydrophobic effect, but self-assembly will occur when the energy of electrostatic repulsion becomes less than that of the hydrophobic attraction. This qualitative concept is similar to the arguments found in the literature on leucine zipper proteins, but there is no convincing evidence that these reasons alone are sufficient to explain why assembly occurs in some cases and not others. The Derjaguin−Landau−Verwey−Overbeek (DLVO) theory quantitatively describes this interaction of forces and allows us to predict how KFE12 should behave when bathed in different media.[21] In this article, we demonstrate experimental corroboration of DLVO theory predictions that the critical coagulation concentrations (CCC) for KFE12 is determined by the anion concentration, that the CCC depends on the valence of the anion to the −6 power, and that KFE12 is a sol at pH below 5 and above 10 but a gel between those pH values.

Materials and Methods

Materials. All chemicals were used as received unless otherwise specified. Biosynthesis grade *N*,*N*-dimethylformamide (DMF) and anhydrous ethyl ether were obtained from EM Science (Darmstadt, Germany). Piperidine 99%, methylmorpholine, and thioanisole 99% were purchased from Aldrich Chemcial Co., Inc. (Milwaukee, WI). Trifluoroacetic acid was ordered from Pierce (Rockford, IL). 1,2-Ethanedithiol was supplied by ICN Biomedicals, Inc. (Aurora, OH). Acetic anhydride, dichloromethane, methyl alcohol, and potassium chloride were obtained from Mallinckrodt Chemical (Paris, KY). Potassium sulfate and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO).

Oligopeptide Synthesis. KFE12 (FKFEFKFEFKFE) was synthesized using Fmoc chemistry on a Protein Technologies PS-3 peptide synthesizer (Ranin Instrument Co., Inc., Woburn, MA) using approximately 0.5 mmol rink amide glutamic acid resin (AnaSpec, Inc., San Jose, CA) and 1.0 mmol t-Boc protected amino acids with 1.0 mmol HBTU activator (AnaSpec, Inc.). Two 20 min deprotection steps in 20% piperidine in DMF were followed by activation of the next amino acid in 0.40 M *N*-methylmorpholine in DMF and a 1 h coupling step as in Wellings and Atherton.[22] The final step consisted of two deprotection steps followed by 20 min of reaction with an excess of acetic anhydride. After drying the resin by washing with dichloromethane and methyl alcohol, we performed cleavage from the resin and protecting groups using a solution of 8.3 mL of trifluoroacetic acid, 270 μL of 1,2-ethanedithiol, and 450 μL of thioanisole for 3 h followed by three precipitations in nearly 50 mL of ethyl ether.[22] After reconstitution in roughly 20 mL of ultrafiltered water (Millipore, Bedford, MA) at 18.2 mΩ, the solution was freeze-dried for several days.

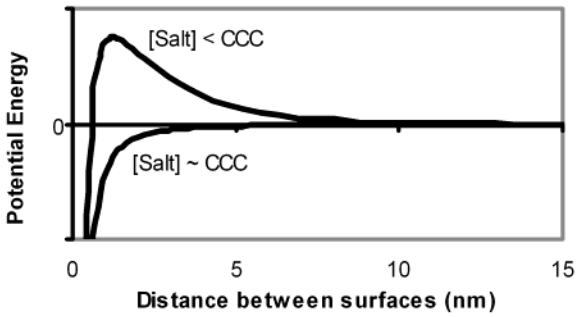

Figure 2. Schematic plot of potential energy vs distance between two charged flat plate surfaces. These curves were obtained by inputting reasonable order-of-magnitude parameter values into the sum of eqs 43 and 48c from Verwey and Overbeek.[23] The curve that exhibits a maximum ([salt] < CCC) was plotted using a Debye length that corresponds to a salt concentration below the CCC. The curve without a maximum ([salt] ~ CCC) corresponds to the potential energy function using the Debye length when the salt concentration is nearly equal to the CCC.

Rheometric Assay for Gelation. Roughly 3 mg of KFE12 powder was mixed with ultrafiltered water to bring the concentration to 1 wt %. Immediately after vortexing for about two minutes and sonicating until the oligopeptide was in solution (~20 min), a 200 μL aliquot was placed on the plate of a rheometer (model AR1000, TA Instruments, Inc., New Castle, DE). A 2 cm diameter, 4° stainless steel cone with truncation at 101 μm was brought, after removal of excess solution, to a height of 101 μm, and a solvent trap was placed around the cone with vacuum grease applied to obtain a liquid tight seal. A test at applied torque of 10 μN·m was performed between 50 and 2.0 rad/s at 25 °C to provide a baseline. The cone was then stopped and, while zero velocity on the cone was maintained, ~30 mL of the appropriate bath solution was added through a port in the top of the solvent trap and nearly 1 mL of vegetable oil was laid over the bath to prevent evaporation. After equilibration for roughly 15 h at 25 °C, the sample was tested over a range of frequencies from 10 to 1.0 rad/s at 2.0 μN·m oscillatory stress. For the experiments shown in Figure 5, the vegetable oil was removed with a micropipet, and then 10 mL of the bath was placed into a centrifuge tube. The pH of this liquid was measured using a pH probe (Orion Research, Inc., Boston, MA) calibrated between pH 4.01 and pH 7.00 using standard buffers (VWR Scientific Products, West Chester, PA).

Results and Discussion

Results were analyzed to determine whether they corroborated predictions from DLVO theory. Figure 2 schematically depicts the potential energy of a system of two charged surfaces as a function of the distances between the surfaces (sum of eqs 43 and 48c in Verwey and Overbeek[23]). The key features of the plot are the activation barrier for the curve at low salt concentration and the sharp decrease in potential energy at small separation distances for both curves.[23] The energy barrier due to electrostatic repulsion kinetically

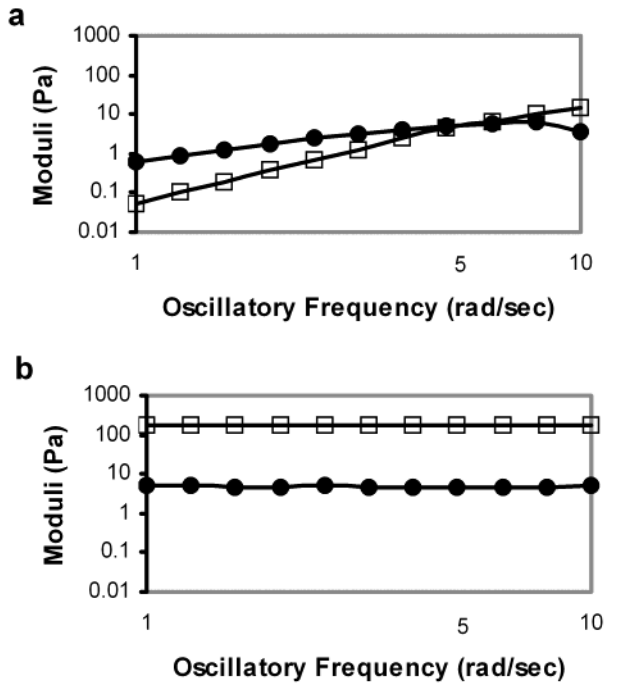

Figure 3. Primary data for gel formation of 1 wt % KFE12 equilibrated with (**a**) 100 μM KCl and (**b**) 1000 μM KCl. The storage modulus (squares), G', and loss modulus (circles), G'', are plotted against oscillatory frequency on log−log scales.

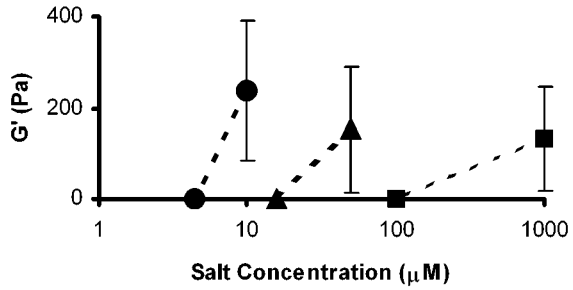

Figure 4. Rheology results for addition of monovalent, bivalent, and trivalent salts. Data for G' at 1 rad/s for KFE12 equilibrated in various concentrations of $K_3Fe(CN)_6$ (circles), K_2SO_4 (triangles), and KCl (squares). The points where G' is near zero are viscous and each of the other points are gels; thus, the dashed lines represent the range over which the CCC exists for each salt. Error bars represent 95% confidence intervals. Error bars for the viscous points are plotted but are smaller than the plotted points.

Table 1. Comparison of Experimental Results with Schulze−Hardy Rule Predictions

salt	anion valence	range of CCC (μM)	valence^{-6}	predicted range (μM)
KCl	1	100−1000	1	100−1000
K_2SO_4	2	15.6−50	0.016	1.6−16
$K_3Fe(CN)_6$	3	4.4−10	0.0014	0.14−1.4

inhibits charged surfaces from assembling; but, if its amplitude is sufficiently decreased (e.g., by screening the charges through the addition of salt), the attraction force becomes dominant causing the surfaces to pack at a distance that we consider to be assembled. We refer to this distance as the minimum because, ultimately, steric repulsion prevents the surfaces from getting any closer and causes the potential energy to approach infinity as the distance between surfaces goes to zero: this effect is not shown in Figure 2.

We can make several predictions about the behavior of KFE12 by using the equations upon which Figure 2 is based. First, at low salt concentration, KFE12 should be kinetically inhibited from forming gels, but at high enough salt concentrations KFE12 should rapidly assemble to form a gel. This prediction is based upon the fact that increasing salt concentration screens the charges on the surfaces which is characterized by a decrease in the Debye length (characteristic length over which charges interact with one another). Sufficient screening causes the amplitude of the kinetic barrier to decrease to less than kT (thermal energy). Experimentally, we found KFE12 at 1 wt % in water (approximately 5.90 mM) to be a viscous solution at low salt concentrations (Figure 3a); but a gel at high salt concentration (Figure 3b). This sol−gel transition was assayed using a rheometer that measures the complex modulus. For a viscous solution, the viscous component of the complex modulus, the loss modulus (G''), decreases with decreasing oscillatory frequency, and the elastic component of the complex modulus, the storage modulus (G'), is low. For gels, G' and G'' are relatively constant with oscillatory frequency, and G' is much greater than zero.[24] These experiments were performed near pH 3 because residual trifluoroacetic acid, from synthesis, causes the solution to be at low pH unless neutralized. The result in Figure 3 is consistent with our hypothesis because increasing the counterion concentration should decrease the amplitude of the activation barrier.[23] DLVO theory defines the critical coagulation concentration (CCC) as the salt concentration at which the amplitude of the activation barrier becomes on the order of kT; thus, above the CCC, KFE12 would no longer remain in the viscous state but would assemble into a gel. The data show that the CCC for 1 wt % KFE12 is between 100 and 1000 μM KCl.

An extension of DLVO theory, known as the Schulze−Hardy rule, provides the second prediction that the CCC will only depend on the concentration and valence of the ion with charge opposite that of the surface charge.[25] The Schulze−Hardy rule, derived using the function shown in Figure 2 at the CCC, predicts in approximation that the CCC will vary inversely with the valence of the counterion to the sixth power.[21] Figure 4 shows the values of G' at 1 rad/s for 1 wt % KFE12 equilibrated in various concentrations of KCl, K_2SO_4, and $K_3Fe(CN)_6$. Since 1 wt % KFE12 is at pH 3, the lysine side chains are positively charged, but the glutamic acid side chains are protonated and thus uncharged. This means that the anions will be the counterions, and the concentration of cation should not effect the CCC. The experimental findings shown in Figure 4 reveal that KFE12 is gelled at 30 μM potassium (in 10 μM $K_3Fe(CN)_6$) but not at 100 μM potassium (in 100 μM KCl); thus, the potassium (and therefore the cation) concentration is not critical at low pH. Figure 4 also shows that CCC for all three salts exist and are different. In Table 1 we present the ranges for the CCC and the predicted values with the range for KCl taken as the reference. This table shows that the experimentally determined ranges for the CCC scale approximately according to the inverse of the valence to the sixth power although the actual power law dependence is measured to be −4.3.

We believe that the underprediction may have several causes including the increasing size of the anion with increasing valence. The Schulze−Hardy rule assumes the counterions are point-sources; but, as the charge becomes dispersed over a larger area, the effect on the electric field becomes less pronounced, which means that the bivalent and trivalent ions which we used will screen the charges less effectively.

The third prediction that we tested was that, in the absence of net charge on the oligopeptide, KFE12 should assemble even in the absence of salt. Since the activation barrier in Figure 2 is present because of the surface charge, bringing the surface (or, in this case, the oligopeptide) net charge to zero should eliminate the barrier. Thus, oligopeptides near pH 7 should self-assemble at all salt concentrations. KFE12 at low pH is positively charged (explained above); however, KFE12 at pH ∼7 will have one negatively charged glutamic acid for each positively charged lysine, resulting in a net charge of zero. Thus, if KFE12 is brought to pH ∼7, DLVO theory predicts that the molecules should self-assemble even without the addition of counterions. Although it was not possible to bring KFE12 to net charge of zero without adding salt, we did accomplish this by adding NaOH. Since we have already demonstrated that the cation was not sufficient to cause gelation, addition of Na^+ ions should not cause gelation. In addition, the OH^- ions will not reach the CCC for a monovalent anion until the solution reaches pH > 11. Thus, we tested this prediction by varying the NaOH concentration in the bath and plotting G' at 1 rad/s vs the pH of the bath in equilibrium with the peptide and vs the calculated fraction of the possible charge on the oligopeptides. This experiment should also exclude the possibility that the anion is acting through some other pathway (e.g., increasing the entropic penalty of exposing hydrophobic side chains) because KFE12 should gel before the anion concentration exceeds 0.1 μM and should become a viscous solution again even as both the anion and cation concentration monotonically increase.

The principles of Donnan equilibrium can be used to calculate the charge density on the oligopeptide after making several simplifying assumptions and measuring the equilibrium pH of the bath solution.[26] Assuming that the pK_a of glutamic acid is 4.5, that the pK_b of lysine is 10.5, that initially there is one trifluoroacetic acid anion for every lysine, and that the Na^+ concentration in the bath is known and constant, electroneutrality is used to relate the ion concentrations in the gel phase.

$$\rho_m + [Na^+] + [H^+] - [TFA^-] - [OH^-] = 0 \qquad (1)$$

Here the charge on the oligopeptide ρ_m is

$$\rho_m = \frac{N_b[H^+]}{K_b + [H^+]} - \frac{N_a K_a}{K_b + [H^+]} \qquad (2)$$

where N_a and N_b are the total concentration of glutamic acid residues and lysine residues in the gel phase, where K_a and K_b are the equilibrium dissociation constants for glutamic acid and lysine, respectively, and where [ion] is the concentration of the respective ions in the gel phase. By equating three activity ratios of similar ions in the gel phase and in the bath phase (dilute concentration allows us to set activity coefficients to unity), and by assuming that the OH^- in the gel maintains equilibrium with the pH in the gel phase

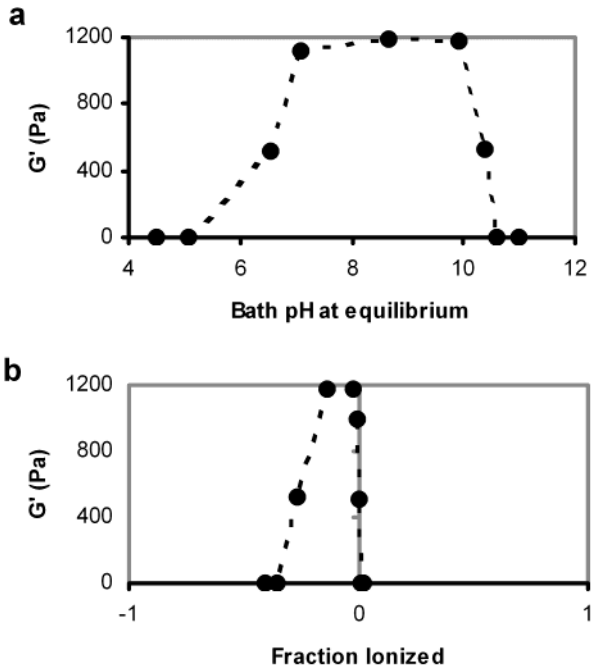

Figure 5. Rheology results for addition of NaOH. (**a**) Data for G' at 1 rad/s for KFE12 equilibrated in various concentrations of NaOH. The pH of the bath at equilibrium was measured and is plotted on the *x*-axis. (**b**) Data from Figure 5a with the *x*-axis converted to fraction of the possible net charge using Donnan Equilibrium calculations. Points where G' at 1 rad/s ≫ 1 represent gels; points near zero represent viscous samples.

$$\frac{[H^+]}{[H^+]_{bath}} = \frac{[Na^+]}{[Na^+]_{bath}} = \frac{[TFA^-]_{bath}}{[TFA^-]} \qquad (3)$$

$$[H^+][OH^-] = 10^{-14} \qquad (4)$$

We were thus able to substitute into the electroneutrality equation (eq 1) for all variables except $[H^+]$ and $[H^+]_{bath}$. $[ion]_{bath}$ is the concentration of the respective ions in the bath at equilibrium. Measurement of $[H^+]_{bath}$ provides a means to solve for $[H^+]$ by iteration of the electroneutrality equation (eq 1). We then substituted this value into eq 2 to find ρ_m and divided by the maximum possible charge to find the fraction ionized.

Figure 5a shows that KFE12 remains viscous at bath pH < 5 (the pK of glutamic acid is near 4.5)[27] and bath pH > 10 (the pK of lysine is near 10.5)[27] but that it forms gels at neutral pHs. Figure 5b gives a useful replot of these data with the *x*-axis converted to fraction of the possible ionization of the oligopeptide; fully ionized (fraction ionized = 1) represents charge density of 5.84 mM. This result indicates that the oligopeptides form gels when charge on the peptide is nearly zero but remain viscous when highly charged, either positive or negative. We expect the peak to be skewed to the negative side of zero because the Na^+ concentrations (cations are the counterions for negatively charged oligopeptides) for the data in this peak are in the 200−800 μM range (due to addition of varying concentrations of NaOH). This is within the range for the CCC for a monovalent counterion.

We do not see a similar offset on the positive side of zero because the concentration of OH^- ions near zero charge (pH $\sim$ 7) on the oligopeptide is in the 0.01−0.1 μM range: much less than the range for the CCC.

Conclusion

In conclusion, we have provided here new data consistent with the hypothesis that self-assembly of KFE12 is regulated by the superposition of van der Waals attraction and electrical double-layer repulsion as quantified by DLVO theory. This not only supports the view that electrostatic specificity over self-assembly conditions is due to like-charge repulsion, but also quantifies the effect electrostatics play in this biological assembly process. Additionally, we anticipate that these results are generalizable to the other members of the family from which KFE12 was derived, oligopeptides that alternate hydrophobic and polar or charged side chains. This is an important advance in gaining a quantitative understanding of why proteins self-assemble under certain conditions but not others, an advance with relevance to several lines of research. In efforts to construct extracellular matrix analogues useful for minimally invasive medicine (our immediate interest), this understanding should allow us to create materials which undergo gelation under specific conditions. For example, a material constructed from synthetic proteins[28] uses the assembly of coiled-coils to achieve a material which is viscous at extreme pH but is gelled at neutral pH (similar in result to Figure 5a). An ideal material would undergo the same transformation starting at 37 °C and pH $\sim$ 7 as a viscous solution and transforming to a gel with only an increase in salt concentration. We believe that the theoretical understanding gained here should aid prediction of oligopeptide sequences that have those properties.

At the same time, in studies of proteins whose self-assembly may lead to tissue pathologies, such as amyloid and prion proteins, this same understanding should aid prediction of interventions for which assembly of harmful plaques could be inhibited. Current research proposes adding soluble molecules which will prevent the prion proteins from adopting pathological conformations; knowledge of why the molecules self-assemble into those conformations may allow rational design of such blocker molecules.[6] Research on simple molecules, like KFE12, will elucidate the driving forces in self-assembly that can then be applied to improve the models of complex proteins such as prions and β-amyloid.

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