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Exploiting Long-Lived Molecular Fluorescence

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Abstract: Fluorophores based on the azo chromophore 2,3-diazabicyclo[2.2.2]oct-2-ene, referred to as fluorazophores, display an exceedingly long fluorescence lifetime. Besides the use in time-resolved screening assays, where the long-lived fluorescence can be time-gated, thereby improving the signal to background ratio, a distinct application of fluorazophores lies in the area of biopolymer dynamics. For this purpose, one chain end is labeled with a fluorazophore and the other one with an efficient fluorescence quencher. The fluorescence lifetime of the probe/quencher-labeled peptide then reflects the kinetics of intramolecular end-to-end collision. Applications to polypeptides are described and control experiments which establish the nature of the quenching mechanism as a diffusive process requiring intimate probe/quencher contact are described.

Keywords: Azoalkanes · Fluorescence · Kinetics · Photochemistry · Peptides

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Werner Nau graduated with a M.Sc. in Chemistry 1992 from St. Francis Xavier University, Canada. His thesis was supervised by D. Klapstein and dealt with molecular spectroscopy (UV, IR, photoelectron) of acyl iso(thio)cyanates. He got his Ph.D. together with W. Adam in 1994 from the University of Würzburg on the EPR and transient absorption spectroscopy of 1,3-cyclopentanediyl diradicals. Thereafter, Werner Nau spent his postdoctoral studies with J.C. Scaiano at the University of Ottawa, where he worked on the mechanistic photochemistry of n,π*-excited states. In 1996, he joined J. Wirz at the University of Basel, where he has started up with an independent research group and became a SNF assistant professor in 2000. In the same year he finished his habilitation. Since the fall semester 2002 he has been appointed as a professor of chemistry at the newly founded International University Bremen.

His research interests lie in the general area of physical organic chemistry and focus on photochemistry, radical chemistry, supramolecular chemistry, and biomolecular chemistry, including both synthetic-preparative, mechanistic, kinetic, and spectroscopic aspects. He has recently introduced a novel class of fluorescent probes, referred to as fluorazophores, which are based on the azo chromophore 2,3-diazabicyclo[2.2.2]oct-2-ene. Fluorazophores are applied as sensors for antioxidants, versatile guest molecules in supramolecular chemistry, kinetic probes for biopolymer folding, and fluorophores for time-resolved screening assays.

Werner Nau has held numerous fellowships, among others a Kekulé and Liebig fellowship of the Fonds of the Chemical Industry, a NATO fellowship, a NSERC International fellowship, and a SNF Profil fellowship. His work has led to the award of several prizes, including the 1999 International Grammaticakis-Neumann prize for photochemistry, awarded by the Swiss Group for Photochemistry and Photobiology, and the 2000 ADUC-Prize, awarded for his habilitation thesis.

Fluorescent probes and sensors are well-established tools in analytical and biological chemistry, spanning such diverse applications as calcium ion detection, cell staining, and polarity sensing [1]. An interesting sub-class of fluorescent probes comprises chromophores with a particularly long fluorescence lifetime, *e.g.* more than 50 ns [2]. Perhaps the simplest yet very important application based on long-lived fluorescence (or generally luminescence) relies on the reliable differentiation of long fluorescence lifetimes from any shorter-lived luminescence components. This is of interest, in particular, for screening assays where fluorescent probes are employed to signal molecular events such as the inhibition of an enzyme by a library of potential drugs. Short-lived emission is ubiquitous and may stem from other additives, sample impurities, biological components, scattered light, the solvent, or sample container materials of cuvettes and microplates.

The shorter-lived components can be eliminated from detection through a time-gate, such that the emission from the long-lived fluorescent label (which serves as the signaling unit) can be selectively detected. This reduces the background during the measurement dramatically. An instructive example is depicted in Fig. 1, which compares the fluorescence decay of a long-lived fluorescent probe (τ = 500 ns) with that of a shorter-lived fluorescing component (τ = 10 ns), with the latter one, however, being much more intense (10^6 times larger preexponential factor). If as usual the integrated fluorescence intensity would be compared through steady-state methods, a 'signal-to-background' of 0.00005:1 would result, which would prevent any useful information to be obtained. If one carries out the experiment in lifetime mode with a time-gate between 200–1000 ns and integrates the areas under the curves, the 'signal-to-background' ratio becomes better than 10000:1, an impressive improvement by more than eight orders of magnitude, which has its underlying reason in the exponential decay kinetics of the fluorescence. This improvement renders the selective detection of long-lived fluorophores in so-called 'time-resolved' screening assays generally an entirely instrumental problem. In particular, the real background may be dominated by detector noise rather than contributions from short-lived emission.

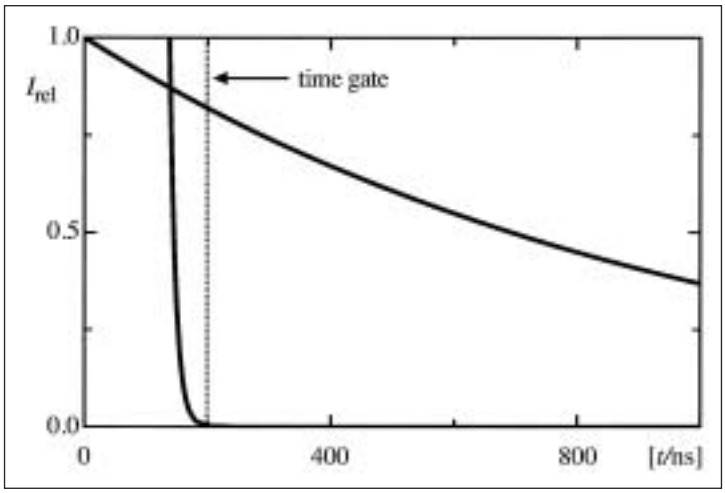
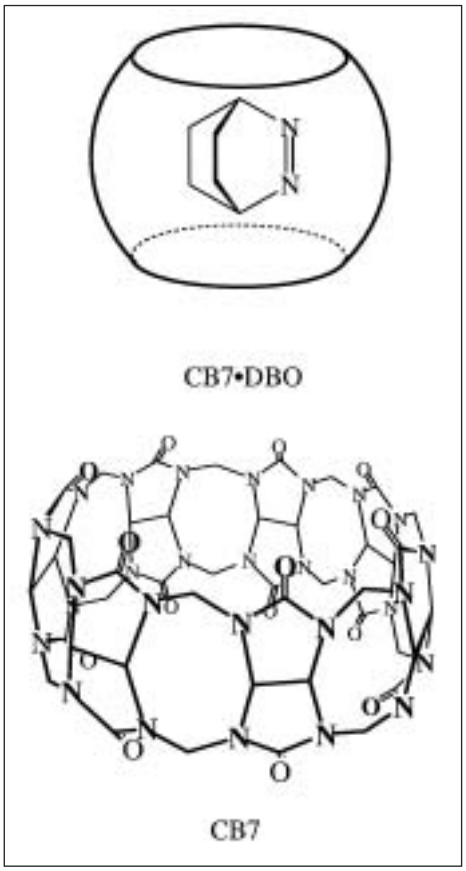
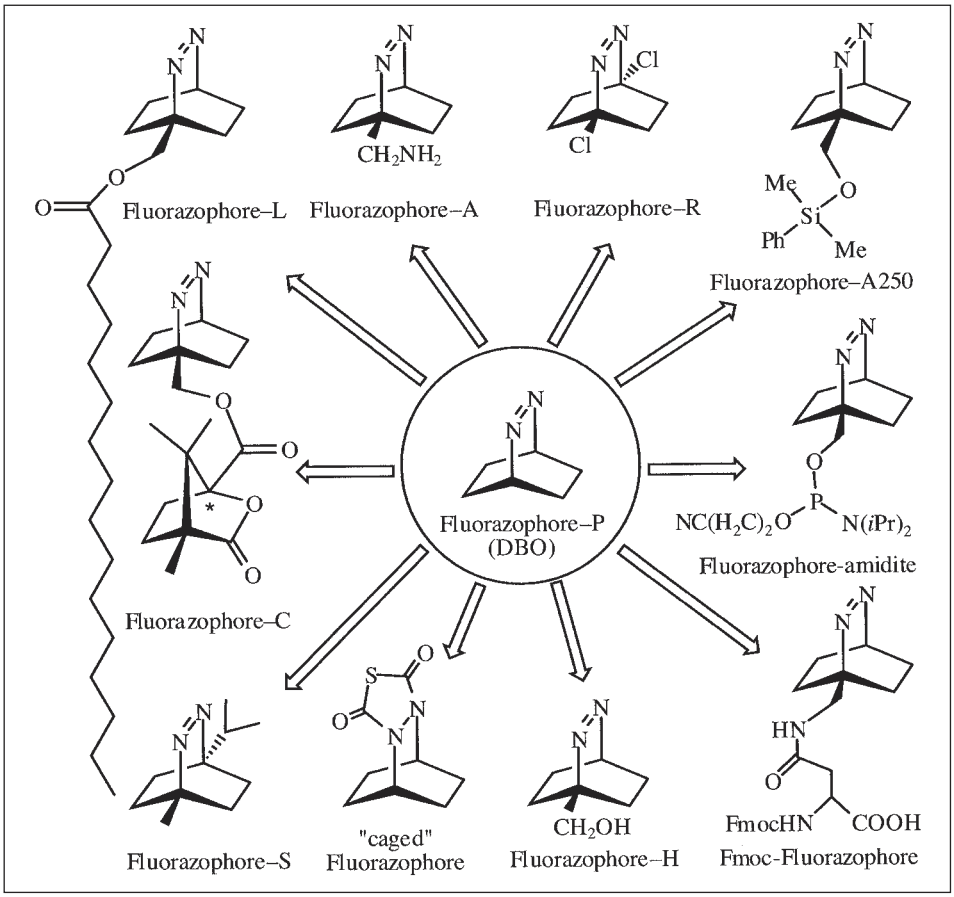


Fig. 1. Comparison of the fluorescence decay of a long-lived fluorescent probe (τ = 500 ns) with that of a shorter-lived component (τ = 10 ns); the shorter-lived component is 10^6 times more intense (relative preexponential factors). A suitable time gate for use in a time-resolved assay is shown at 200 ns.

Relatively few organic molecules display lifetimes in this long time regime, with azoalkanes derived from 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) displaying the longest fluorescence lifetime in solution [3]. The record for the longest fluorescence lifetime of an organic chromophore in solution lies currently at 1.03 µs (in aerated H_2O!) [4] and is held by the supramolecular complex (CB7•DBO) between the parent azoalkane and cucurbit[7]uril (CB7), a barrel-like organic host molecule [5]. Over the past six years, we have investigated this interesting chromophore in great detail and have prepared several DBO derivatives, which we refer to as fluorazophores ('fluorescent azo chromophores'). Some of the investigated derivatives are shown in Scheme 1.





Scheme 1

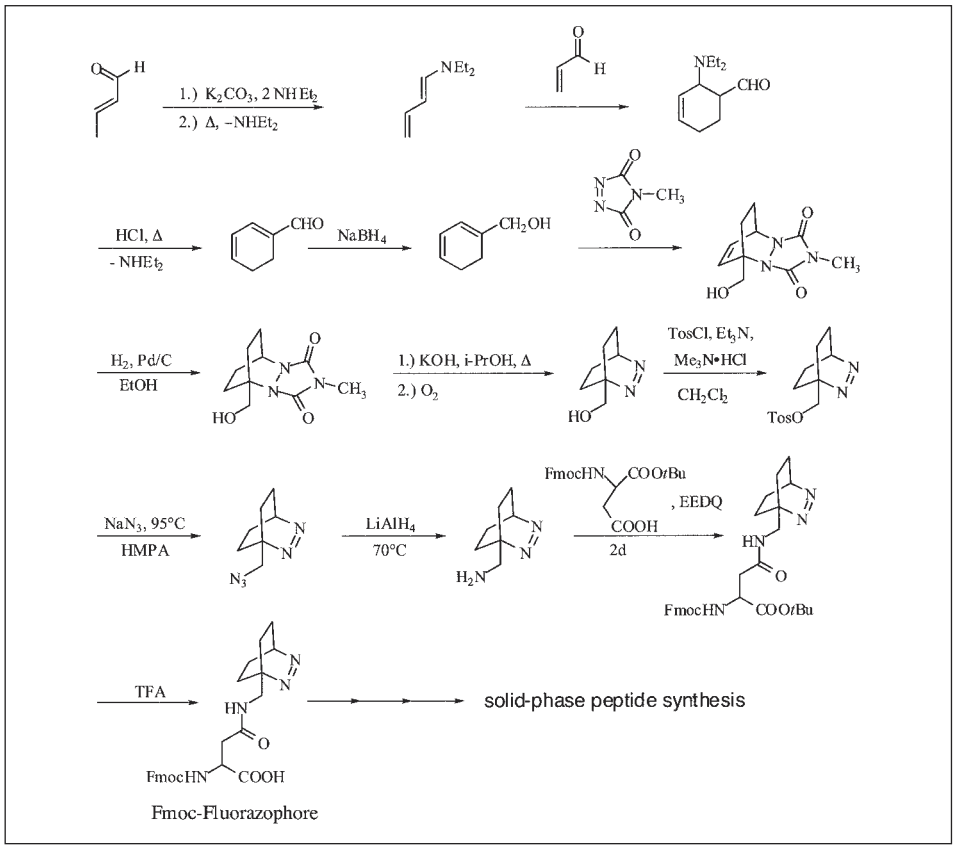
A representative synthetic sequence to obtain Fmoc-Fluorazophore is shown in Scheme 2, which has been scaled up to afford typically 3 g of Fmoc-DBO in an overall yield of 10%, sufficient for the commercial synthesis of up to 30 polypeptides (10 mg scale).

The intriguing photophysics of fluorazophores has been worked out in great detail [6–11] and the fascinating quenching pathways have been investigated through a combination of experimental and theoretical methods [12–14]. On the more applied side, fluorazophores have proven to be useful as sensors for antioxidant activity, both in solution [15][16] as well as in membrane-mimetic systems [17], as probes for measuring the kinetics of association with supramolecular systems [18][19], as tools to investigate the geometries of cyclodextrin inclusion complexes by means of circular dichroism [20][21], and as probes for the polarizability inside molecular container molecules [22].

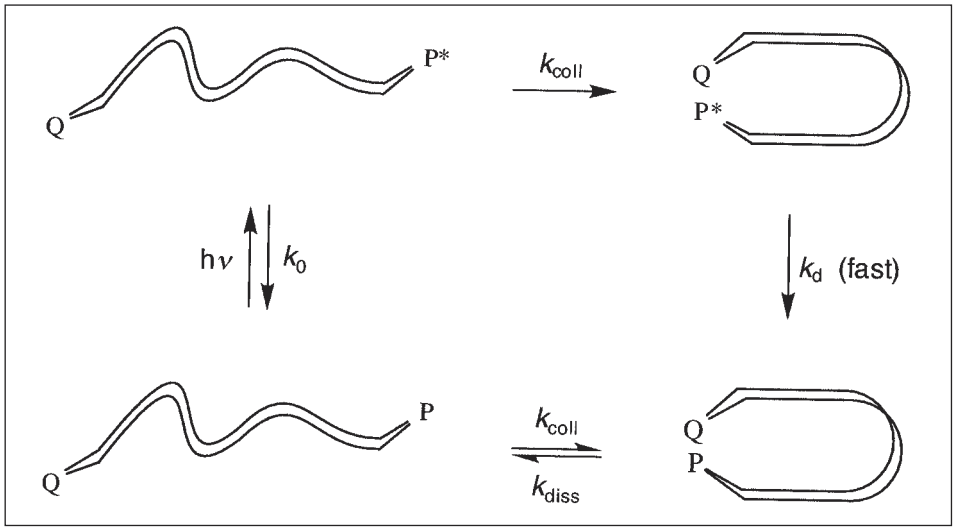
Most recently, we have employed fluorazophores to measure the kinetics of end-to-end collision in biopolymers (k_{coll} in Scheme 3), including peptides [23][24], and oligonucleotides [25]. For this purpose, the fluorazophore (P) is attached to one end of the biopolymer and an efficient (nearly diffusion-controlled) fluorescence quencher (Q) is attached to the other side; in peptides this quencher moiety is usually tryptophane and in oligonucleotides guanine. The kinetics of fluorescence quenching can then be equated to the rate of end-to-end collision, which has proven difficult to obtain accurately by alternative techniques.

Keeping in mind that alternative methods have other advantages [26][27], the fluorazophore approach presents, arguably, the most sensitive and most accurate tool for measuring end-to-end contact in biopolymers known to date. Note that this application is made possible by the exceedingly long fluorescence lifetime, which allows the fluorazophore to 'wait' sufficiently long until it is being approached (and immediately quenched) by the other end; this diffusive approach of the chain ends takes 10 ns to 1 μs in aqueous solution. The lifetime of fluorazophores (*ca.* 505 ns in D_2O under air) is therefore ideally suited for investigations in this time regime.

Being able to measure the absolute rates for the motions within biopolymers or at least knowing the time scale for these processes is of fundamental importance for understanding the mechanism of protein folding [28], for predicting the kinetics of intramolecular reactions in biopolymers (formation of hydrogen bonds, cystine bridges, proton transfer, electron transfer)



Scheme 2

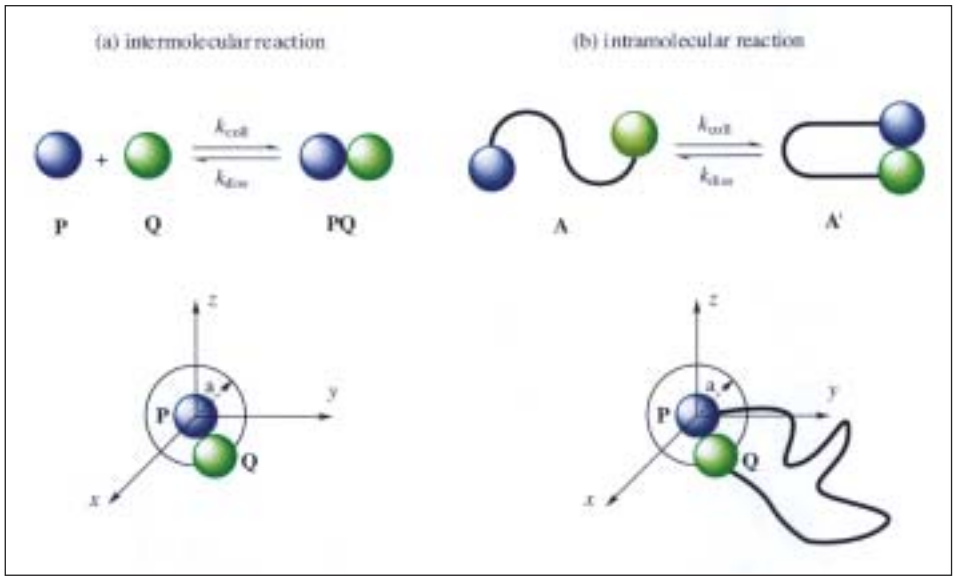


Scheme 3

[29], and for understanding protein domain motions [30]. The size of biopolymers and the effects of solvation, including salt effects, still present a major challenge to computational chemistry, which demands experimental data. The latter, in turn, may provide invaluable benchmark values for the calibration of theoretical models [31].

A simple problem already arises if one attempts to predict the time scale of diffusion-controlled end-to-end collision in (bio)polymers from the available rates for diffusion-controlled intermolecular reactions. To allow a comparison, it is useful to compare the probability for intramolecular and intermolecular encounter complex formation between two fragments.

Consider Scheme 4a, the intermolecular case. We assume an ideal solution with no enthalpic interactions between the probe (P) and the quencher (Q) molecules. The concentration of the encounter complex, C_{PQ}, can be obtained according to Eqn. (1) with V_e being the volume of the encounter complex with radius a (spherical approximation). $C_P{}^0$ and $C_Q{}^0$ are the total concentrations of P and Q. The 'equilibrium constant' for encounter complex formation is then given by Eqn. (2), assuming that the concentration of molecules in contact is



Scheme 4

small (dilute solution, *i.e.* $C_P \approx C_P^0$ and $C_Q \approx C_Q^0$). It follows that the equilibrium constant for encounter complex formation (in units of m^3mol^{-1}) equals the volume of the encounter complex multiplied with the Avogadro constant (N_a). This in turn corresponds, due to the absence of enthalpic effects, to the loss of entropy associated with the formation of the encounter complex, *i.e.* $\Delta_{coll}S^0 = R\ln(1000K_{inter}C^0)$, where the factors 1000 and C^0, the standard concentration in *M*, are added to give a dimensionless equilibrium constant.

$$C_{PQ} = C_P^{\,0} N_a V_e C_Q^{\,0} = C_P^{\,0} C_Q^{\,0} N_a \frac{4}{3}\pi a^3 \quad (1)$$

$$K_{inter} = \frac{C_{PQ}}{C_P C_Q} \approx \frac{C_{PQ}}{C_P^{\,0} C_Q^{\,0}} = \frac{4}{3}\pi a^3 N_a \quad (2)$$

The intramolecular case, Scheme 4b, describes the pertinent situation for encounter complex formation within a biopolymer chain labeled with a probe (P) and a quencher (Q) at opposite ends. For simplicity, we assume that the chain does not introduce additional interactions between probe and quencher except to restrict the distance by which they can diffuse apart (ideal chain, Gaussian chain). Like for the intermolecular case, we assume no enthalpic interactions between the probe and the quencher residues. The fraction of chains with the two ends in contact, ($C_{A'}$), can be obtained in this case from the distribution function (g) in Eqn. (3), which has analytical solutions for a very long chain ($N >> 1$, with N the number of chain segments) and for the shortest chain ($N = 2$). r is taken as the distance between the chain ends and b equals the length of an individual chain segment. The 'equilibrium constant' for end-to-end encounters is then given by Eqn. (4), which assumes for the case of a very long chain ($N >> 1$) that the concentration of molecules in contact is small ($C_A \approx C_A + C_{A'}$). Again, this relates directly to the loss of entropy associated with the formation of an end-to-end encounter complex within an ideal chain, *i.e.* $\Delta_{coll}S^0 = R\ln K_{intra}$.

$$g(r) = \left(\frac{3}{2\pi\langle r^2\rangle}\right)^{3/2} \exp\left(-\frac{3r^2}{2\langle r^2\rangle}\right) \text{ with } \langle r^2\rangle = Nb^2 \text{ for } N >> 1$$
$$g(r) = \frac{1}{8\pi r b^2} \text{ for } N = 2 \quad (3)$$

$$K_{intra} = \frac{C_{A'}}{C_A} \approx \frac{C_{A'}}{C_A + C_{A'}} = \int_0^a g(r)4\pi r^2 dr \approx \frac{4}{3}\pi a^3 \left(\frac{3}{2\pi\langle r^2\rangle}\right)^{3/2} = \frac{4}{3}\pi a^3 \left(\frac{3}{2\pi N b^2}\right)^{3/2} \text{ for } N >> 1$$
$$K_{intra} = \frac{C_{A'}}{C_A} = \frac{\int_0^a g(r)4\pi r^2 dr}{1 - \int_0^a g(r)4\pi r^2 dr} = \frac{(a/b)^2}{4 - (a/b)^2} \text{ for } N = 2 \quad (4)$$

The kinetics of end-to-end contact formation for the intramolecular reaction can be related to that of the intermolecular reaction (Eqn. (5)) if one reduces the equilibrium constants to ratios of microscopic rate constants and considers further that the elementary rate of dissociation of the encounter complex must be identical for both species within the approximations made ($k_{diss}^{intra} = k_{diss}^{inter}$). Recall, in particular that the only function of the chain for the intramolecular case is to limit the distance between probe and quencher.

$$\frac{K_{intra}}{K_{inter}} = \frac{k_{coll}^{intra}/k_{diss}^{intra}}{k_{coll}^{inter}/k_{diss}^{inter}} = \frac{k_{coll}^{intra}}{k_{coll}^{inter}} \quad (5)$$

Use of the respective expressions for the equilibrium constants for intermolecular and intramolecular encounter complex formation then affords Eqn. (6). If one further expresses the intermolecular collision rate constant through the relationship between the intermolecular diffusion rate constant and the diffusion coefficient ($k_{coll}^{inter} = 4D\pi aN_a$), one obtains Eqn. (7), with D being the mutual intermolecular diffusion coefficient.

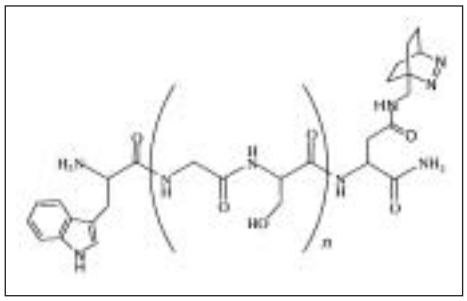
Eqn. (7) provides the ideal relationship between the unknown rate constant for intramolecular end-to-end collision and the known (diffusion-controlled) rate constant for an intermolecular probe-quencher pair. Accordingly, the rate of end-to-end collision in a biopolymer increases linearly with the diffusion coefficient and size of the encounter complex (radius a), it depends inversely on the cubed chain segment length (b), and it decreases with increasing chain length (N) with the characteristic exponent of –3/2. Eqn. (7) has been derived in a different context by Szabo, Schulten, and Schulten through an analysis of the first passage time of end-attached groups based on a modified Smoluchowski equation [32][33].

$$\frac{k_{coll}^{intra}}{k_{coll}^{inter}} = \frac{\frac{4}{3}\pi a^3 \left(\frac{3}{2\pi N b^2}\right)^{3/2}}{\frac{4}{3}\pi a^3 N_a} = \frac{1}{N_a}\left(\frac{3}{2\pi N b^2}\right)^{3/2} \text{ for } N >> 1 \quad (6)$$

$$k_{coll}^{intra} = k_{coll}^{inter} \frac{1}{N_a b^3}\left(\frac{3}{2\pi}\right)^{3/2} N^{-3/2} = \frac{4D\pi a N_a}{N_a b^3}\left(\frac{3}{2\pi}\right)^{3/2} N^{-3/2} = \sqrt{\frac{54}{\pi}}\frac{Da}{b^3} N^{-3/2} \text{ for } N >> 1 \quad (7)$$

To apply Eqn. (7), one may use commonly accepted intermolecular diffusion coefficients of 10^{-5} cm^2s^{-1} in water (the mutual diffusion coefficient in Eqn. (7) corresponds to twice this value), a van-der-Waals reaction radius of 5 Å, and a chain segment length of 5 Å (typical for one peptide unit). The resulting estimates for the intramolecular collision times ($1/k_{coll}$) are 0.2 ns for $N = 4$ and 2.7 ns for $N = 20$. This means that end-to-end contact formation in biopolymers in solution may occur as fast as several ns according to the simplest theoretical framework. These theoretical rates can now be compared with the experimental results obtained from the fluorazophore probe/quencher technique in synthetic polypeptides.

Our initial experimental study focused on the length dependence of the end-to-end collision rates in peptides with the general structure Trp-(Gly-Ser)$_n$-DBO-NH_2 (structure below); these peptides are water soluble and presumed to be 'structureless' according to a previous study [26]. This is an important requirement to apply theories based on Gaussian chain behavior [34][35].



The fluorescence decays of all peptides as recorded with the time-correlated single photon counting technique were strictly monoexponential (Fig. 2). The resulting fluorescence lifetimes (τ) for the peptides with different length are listed in the Table and range from 10–75 ns. Subject to the assumption of diffusion-controlled quenching, the collision rates can be directly obtained through a correction for the inherent fluorescence lifetime (τ_0) according to Eqn. (8). The data for the collision rate constants demonstrate that end-to-end contact formation in short polypeptides may occur as fast as 10 ns in water, significantly faster than previous estimates of rates for peptides with the same length, but also substantially smaller than expected from the ideal-chain behavior according to Eqn. (7) (see above). Presumably, the diffusion coefficients of the peptide chain ends are much smaller than those of the free probe and quencher; the use of smaller diffusion coefficients in Eqn. (7) than the intermolecular ones would bring the theoretical data much closer to the experimental ones [36].

$$k_{coll} = 1/\tau - 1/\tau_0 \qquad (8)$$

Table. Fluorescence lifetimes and end-to-end collision rate constants for Trp–(Gly–Ser)$_n$–DBO–NH_2 polypeptides

n	*N*[a]	τ / ns[b]	k_{coll} / 10^7 s^{-1}[c]
0	2	23.3	4.1
1	4	14.3	6.8
2	6	19.5	4.9
4	10	30.5	3.1
6	14	45.6	2.0
10	22	74.4	1.1

[a]Number of peptide units between probe and quencher. [b]Fluorescence lifetime in aerated D_2O at 23 °C measured by time-correlated single photon counting. [c]Obtained from Eqn. 8 with τ_0 = 500 ns.

The dependence of the collision rates on the chain length as derived in Eqn. (7) predicts a linear increase of the logarithmic collision rates with the logarithm of the number of chain segments (*N*). The corresponding plot (Fig. 3) for the experimental data shows that this linear relationship is not observed. Instead, one obtains a plot with a strong negative curvature. Moreover, the theoretical slope [34][35] of –3/2 is only reached for the longer chains. These variances indicate deviations from the ideal behavior, which are presumably related to effects of chain stiffness, which impose an increased internal friction for end-to-end collision in the short chains [36].

The interpretation of the fluorescence lifetimes in terms of end-to-end collision rate constants requires a collision-induced fluorescence quenching, *i.e.* probe and quencher must come into van-der-Waals contact (2–3 Å distance) for quenching to occur. This is naturally fulfilled for quenching by hydrogen atom transfer or exciplex-induced quenching, which are the two prototypal quenching mechanisms of the DBO chromophore [3][6][8][13][14][37–39]. However, quenching by electron transfer, which presents an alternative quenching mechanism, could operate through bond (superexchange mechanism); it could also occur over a considerable distance through space or through the solvent (up to 5–8 Å) and must therefore be excluded [40]. The same applies for Dexter-type triplet energy transfer, which has been employed in other intramolecular probe/quencher pairs to assess end-to-end contact formation [26][41][42]. Quenching over larger distances than van-der-Waals contact would result in a continuum of distance-dependent rate constants, which could not be analyzed in terms of a diffusion-controlled collision

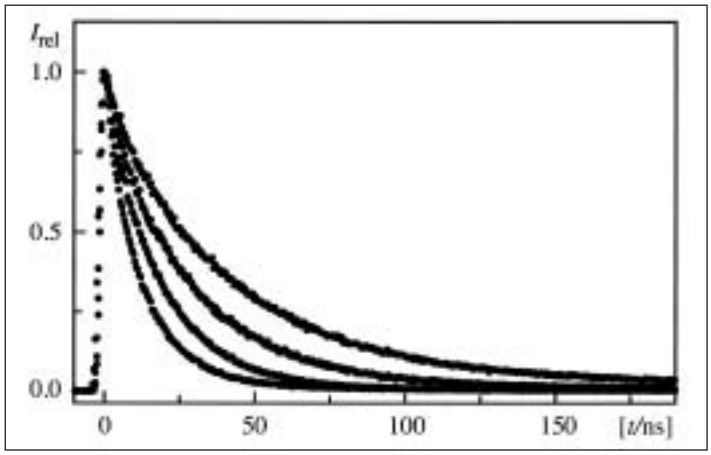


Fig. 2. Fluorescence decays (time-correlated single photon counting, normalized intensity) of Trp–(Gly–Ser)$_n$–DBO–NH_2 polypeptides (n = 1, 2, 4, and 6). The lowest trace corresponds to n = 1, the uppermost one to n = 6.

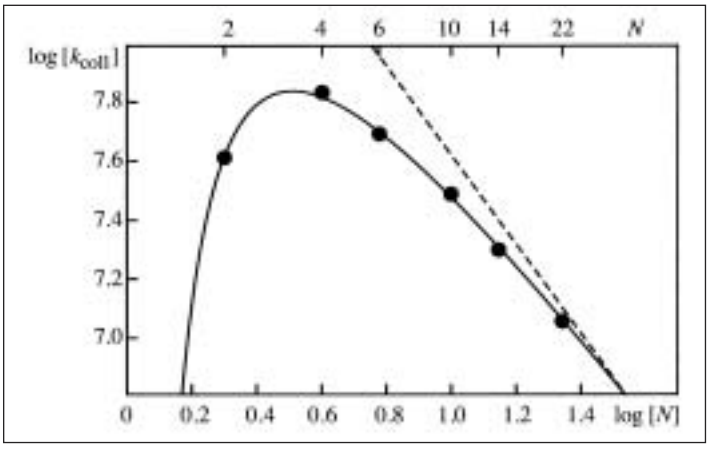


Fig. 3. Double-logarithmic plot of the end-to-end collision rate constants (k_{coll}) of Trp–(Gly–Ser)$_n$–DBO–NH_2 polypeptides *versus* the peptide length, taken as the number of intervening peptide units (*N*). The dashed line has a slope of –3/2 and is shown to illustrate the deviation from the theoretical behavior (Eqn. 7).

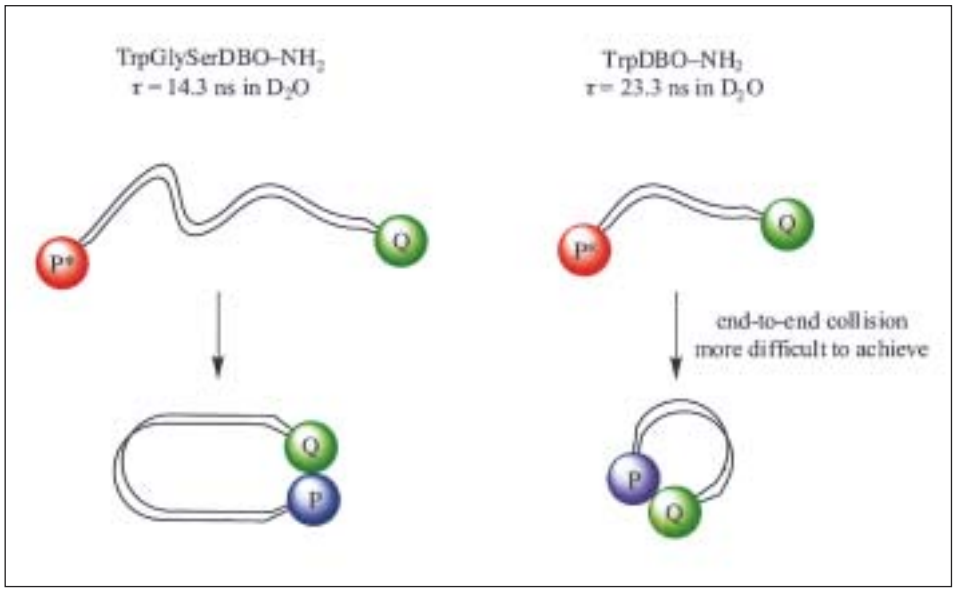
process. In this case, quenching presents no longer an elementary reaction to which a single rate constant can be assigned. In fact, fluorescence resonance energy transfer, which operates over even larger distances, has proven inapplicable to obtain the pertinent elementary rate constants [26][43].

In view of the possible complications due to distance-dependent quenching rates, it appeared compulsory to establish experimentally that quenching through bond or through the solvent do in fact not apply for the DBO/Trp probe/quencher pair. We have therefore performed a series of carefully designed control experiments (Schemes 5–7). Control experiments of this type are strongly recommended to establish alternative methods for assessing end-to-end contact formation, in particular if triplet energy transfer or electron transfer (both of which are candidates for distance-dependent quenching rates) are the postulated quenching mechanisms.

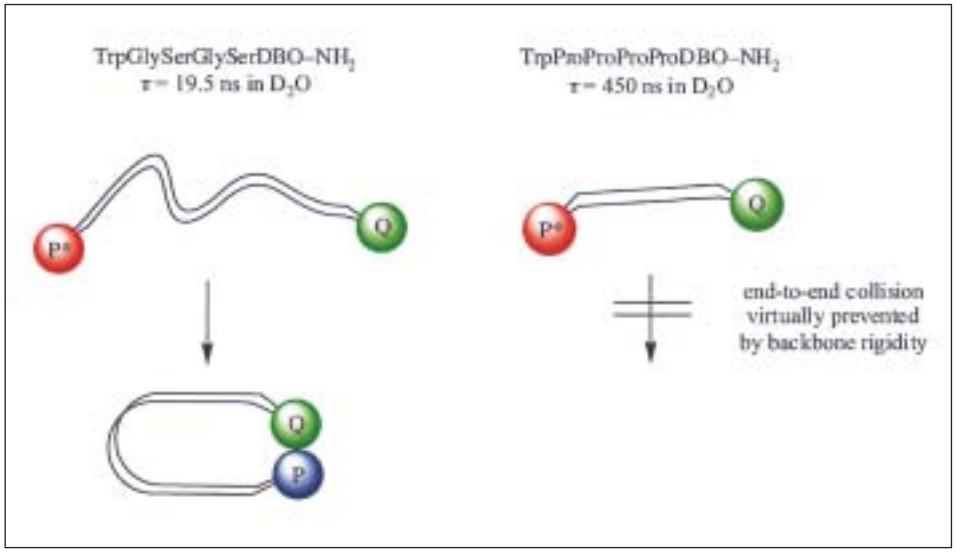
In the first experiment (Scheme 5), we have compared the fluorescence lifetime of the shortest peptide, in which probe and quencher are directly attached, with the longer ones. The lifetime of the shortest peptide is in fact longer than for the next longer one (Table), which speaks strongly against a through-bond quenching mechanism, but can be understood in terms of internal friction (steric hindrance effects) [36]. We encounter this effect in daily life: It is more difficult to make a knot in a very short rope than in a longer one.

In the second experiment (Scheme 6), we have exchanged the presumably flexible amino acids glycine and serine in the backbone of the peptide by rigid cyclic proline spacers. The proline peptide has a much longer lifetime than the glycine-serine one, close to the lifetime in the absence of quencher (505 ns in D_2O). This suggests that quenching through bond is unlikely since the number of bonds remains identical in both species. The effect of rigidifying the backbone provides also strong evidence that it is the diffusion between the chain ends which is decisive for the quenching process. Incidentally, it should also be mentioned that any increase in the solvent viscosity, as it can be achieved, for example, through the addition of denaturants like urea (5 M) or guanidinium chloride (6 M) also decreases the end-to-end collision rates of flexible DBO/Trp polypeptides, consistent with a diffusive process.

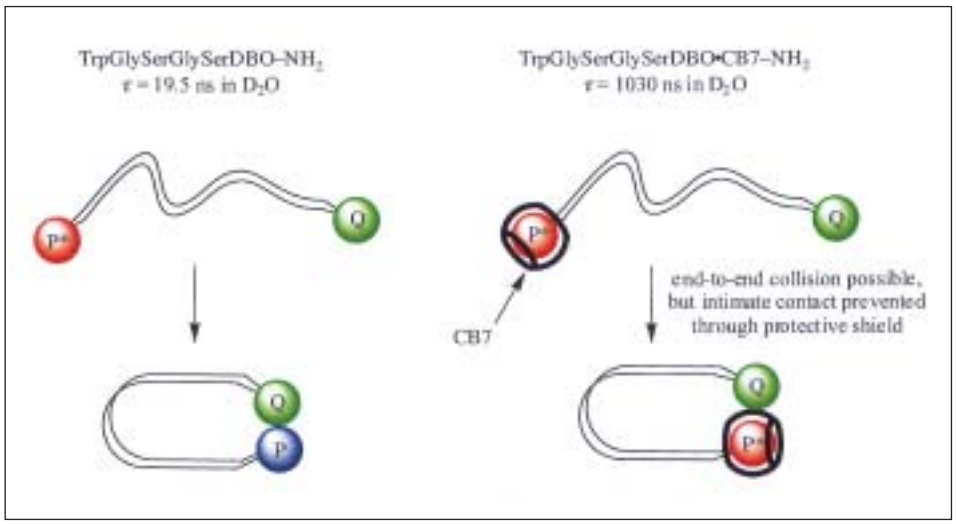
In the third experiment (Scheme 7), we have left the peptide backbone unchanged, but have added cucurbit[7]uril (CB7) to the aqueous solution of the peptide. As demonstrated by NMR experiments, CB7 complexes selectively and quantitatively the



Scheme 5



Scheme 6

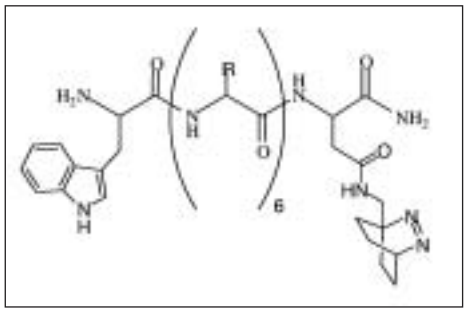


Scheme 7

DBO chromophore ($K = 4 \times 10^5\ M^{-1}$) and thereby provides a 'protective shield' around the chromophore.[22] This shield prevents van-der-Waals contact with the quencher, which is still free to diffuse since solvent and peptide backbone have remained unchanged. The resulting lifetime of the complexed peptide was found to be 1.03 µs, suggesting that the quencher is not able to quench the excited probe at all (the longer lifetime than in D_2O results from the exclusion of oxygen and the solvent from the cavity). This result provides strong evidence for the view that quenching requires intimate contact. If quenching would occur through space or through the solvent it should have also been mediated through the supramolecular wall.

It follows from the control experiment in Scheme 6, that the fluorescence lifetimes of DBO/Trp end-labeled peptides are a quantitative measure of the flexibility or rigidity of the peptide backbone. We have therefore most recently synthesized a series of random-coil peptides, in which Trp and DBO are separated by a sequence of identical amino acids (see structure below) [24]. Each peptide has a characteristic fluorescence lifetime, which can be interpreted in terms of the conformational flexibility, which a particular amino acid imposes on the backbone. This allows one to define *a conformational flexibility scale for amino acids in peptides*. The following order of flexibility applies, where glycine gives rise to the most flexible peptide and proline produces the most rigid one:

Gly > Ser > Asp, Asn, Ala > Thr, Leu > Phe, Glu, Gln > His, Arg > Lys > Val > Ile > Pro



In summary, the intramolecular fluorescence quenching of fluorazophores provides a distinct tool for investigations in the area of biopolymer dynamics. Future studies will involve oligonucleotides, larger, structured peptides, mutation effects, the determination of activation energies, and the transfer of the kinetic results to applications in high-throughput screening technology, where the long fluorescence lifetime provides the additional advantage of suppressing background fluorescence through time-resolved detection (see Fig. 1).

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