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ORIGINAL CONTRIBUTION

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2-(*p*-toluidino)-6-naphthalene sulfonate as a fluorescent probe for flocculation studies of cationic potato amylopectin and nanosized silica particles 1. 2-*p*-(toluidino)-6-naphthalene sulfonate binding to cationic amylopectin

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Abstract 2-(*p*-toluidino)-6-naphthalene sulfonate (TNS) is a probe that fluoresces strongly when bound to certain proteins and polymers, but weakly in aqueous solution. Absorption and fluorescence spectroscopy were used to study the interaction of TNS with native amylopectin potato starch (NApS) and cationized amylopectin potato starch (CApS) in aqueous solution. The anionic TNS binds to CApS at a single type of binding site, with an affinity which has both electrostatic and nonelectrostatic contributions (including hydrogen bonding), whereas binding to NApS occurs at the same type of site but only by nonelectrostatic means. The affinity to CApS decreases strongly with increasing salt concentration, due to screening of the electrostatic attraction, whereas with NApS increasing salt concentration slightly enhances the binding affinity, most likely due to screening of a weak repulsive interaction between TNS and phosphate residues on NApS. The association constant for binding of TNS to CApS in 5 mM NaCl is 110 ± 20 M^{-1}. This comparatively weak binding makes TNS a useful probe in kinetic investigations of the flocculation of anionic silica particles by CApS.

Key words Retention aids – Flocculation – Flourescence – 2-(*p*-toluidino)-6-naphthalene sulfonate – Starch – Silica

Introduction

Flocculation is a process with significance in many fields, such as purification of waste water and in the papermaking industry. There are many studies available on the flocculation between particles and polymers [1]. In the majority of cases the particles are large compared to the radius of gyration of the polymers [1], and the particle surface can be considered as a surface where several polymer molecules can be attached. The reverse case, where the particle radius is less than the radius of gyration of the macromolecule, has been less frequently studied [2, 3]. Both cases can be considered as bridge flocculation. In the former the small polymers bridge the large particles and in the latter the large polymer bridges a number of small particles. Another way to look at the latter case is to consider the polymers as the kinetic units and the particles as the bridge-forming agent. Most of the studies consider equilibrium flocculation between polymers and particles [1–3] or complexation between macroions [4]. The kinetics of complexation [5] and flocculation [6, 7] have been less frequently studied.

Starch is the second most abundant polysaccharide in nature after cellulose and is composed of the linear amylose and the branched amylopectin. Cationic potato starch (CApS) [8] has proved to be a promising flocculation agent in paper making, which has stimulated studies of its properties, such as solubility [9] and interaction with salt and silica particles [10–12]. Nanosized silica particles (NSP) are used together with CApS in the paper industry to improve retention, dewatering and dry strength of the paper sheet [13]. The combina-

tion of CApS and NSP is very efficient as a retention and dewatering aid but the mechanisms by which they operate are not fully understood.

The flocculation processes as they occur in the wet-end of the paper machine are in general difficult to study because they involve a large number of substances such as fibers, fillers, retention aids, fines, bacteria, slime, humic substances and simple electrolytes. The flow in the paper machine is also very turbulent and the relevant processes occur over a wide range of timescales from seconds and shorter. In order to simplify the system and to focus on the interaction between fewer components on appropriate timescales we have studied CApS and anionic NSP using stopped-flow techniques [10]. So far turbidity has been detected, where most of the light scattering is due to the particles. The turbidity increases as the CApS bring about an inhomogenous distribution of particles, both as a consequence of the binding to the polymer and because such particle-polymer complexes form flocs and/or change their conformation. The presence of several contributions made interpretations of the turbidity kinetics ambiguous. It was therefore deemed interesting to follow the flocculation by a second method, in order to try to distinguish these processes.

The charge density of a cationic polymer can be measured by titration with an anionic polyelectrolyte of known charge. The formation of the interpolyelectrolyte complex can be monitored by adding the fluorescent probe 2-(*p*-toluidino)-6-naphthalene sulfonate (TNS) to the cationic polyelectrolyte [14]. As the polymer of opposite charge is added, the negatively charged TNS is released from the polyelectrolyte and its fluorescence decreases because the quantum yield is comparatively low for TNS in water. By titrating until all TNS molecules are released a break point is obtained, the position of which is related to the charge density of the polymer. As the TNS-modified cationic polymer binds the oppositely charged particle, the fluorescent probe is released and the fluorescence decreases. Ideally the TNS fluorescence thus reflects the initial process of particle binding to the polymer, as opposed to, for instance, changes in polymer conformation, which should be helpful in distinguishing between possible mechanisms [10].

This strategy requires that the interactions between the fluorescent probe TNS and the various components of the system have been characterized. The interaction between TNS and CApS is the subject of the present paper, which deals with the mode and strength of binding of TNS to CApS and native amylopectin potato starch (NApS) and how these aspects are affected by the ionic strength. The results are used in another paper [15], which deals with the actual flocculation between NSP and TNS-modified CApS.

Materials and methods

Materials

The Potassium salt of TNS was purchased from Sigma. NApS and CApS were donated by Lyckeby Stärkelsen (Kristianstad, Sweden).

Cationic potato amylopectin

Amylopectin is normally the dominant component in the starch granule [16], and has a molecular weight of about 10^7–10^9 g/mol while amylose has a molecular weight of about 10^5–10^6 g/mol [16]. The CApS used here has 4% of the monomers carrying one unit charge and has a radius of gyration of about 300 nm (at 0.1 mM salt concentration), while the NApS has a radius of gyration of about 200 nm [17].

Amylopectin consists of glucose units connected with $\alpha(1,4)$ bonds in the linear parts and the branching points are $\alpha(1,6)$ bonds [18]. About 4% of the links are branching points [18, 19]. It has been found through enzymatic degration studies combined with HPLC that the amylopectin molecule has a bimodal distribution of chain lengths, with the longer average chain length being 48–65 glucose units and the shorter average chain length being 16–20 glucose units [20, 21]. The different chains are further divided into A, B and C chains [22]. The C chains carry the free reducing end group and are connected with B chains that carry other B chains and/or A chains. The A chains carry no other chains. The A chains correspond to the shorter average chain length while the B chains corresponds to the longer average chain length [21]. These data have been interpreted as amylopectin having highly branched regions alternating with regions with linear polymer strands. It has been found that even if the amylopectin is highly charged (degree of cationization of about 0.3 charges/monomer) the polymer coils only become slightly larger, and without any drastic change in the segment density or internal structure [23, 24]. This is in contrast to linear polymers where coil sizes increase significantly as the polymer becomes more strongly charged [24]. The reason for this difference could be that amylopectin has a rather compact core surrounded by an outer ''hairy layer'' of A chains. It has also been found that the amylopectin coil expansion due to the cationic charges is screened by addition of a few millimoles of salt [10, 12, 25].

Preparation of the solutions

All solutions were prepared with doubly deionized water. The amylopectin (0.20 g) was dissolved in water (100.0 ml) at 96–98 °C under gentle stirring for 30 min and was cooled to room temperature. The final concentration was set by adding water. TNS and NaCl were added in appropriate amounts, and the solutions were degassed for 30 min, as this was the standard procedure in the stopped-flow studies [15].

Spectroscopic measurements

Absorbance spectra were recorded on a CARY 2300 and fluorescence spectra were recorded on a SPEX fluorolog τ2. Unless stated otherwise the excitation wavelength was 315 nm. Corrections for inner-filter effects were not necessary at the low absorbances used. All measurements were carried out at room temperature and at pH 8.

Results

The absorbance and excitation spectra of CApS-bound TNS, using a 460-nm emission wavelength, are shown in

Fig. 1a. The emission spectra of TNS in water and in the presence of CApS and NApS in 5 mM NaCl are compared in Fig. 1b. The most notable difference between CApS and water is that TNS emits about 20 times more strongly in the presence of the amylopectin. We also note that there is a blueshift of the emission maximum (λ_{max}) from 525 nm in water to 460 nm when CApS is added. A very similar blueshift is observed when NApS is added instead, but the fluorescence is less enhanced, reaching about 30% of the intensity with CApS. None of the carbohydrate compounds exhibited any fluorescence in the wavelength range examined, but there was a detectable polymer scattering contribution.

The changes in TNS emission properties reflect the formation of a complex between TNS and the polysaccharides, and this was used to characterize the complex formed. An important observation is that in both cases the shape of the emission spectra was independent of the excitation wavelength ($\lambda_{ex} = 315$ nm and 350 nm; results not shown). This shows that the fluorescence is dominated by one emitting species: the bound form.

In order to investigate the electrostatic properties of the complex between TNS and the amylopectins, emission spectra of TNS in the presence of CApS or NApS were recorded in solutions with different concentrations of NaCl. The spectral shapes at different ionic strengths were very similar (not shown), but the fluorescence intensity at λ_{max} (460 nm for both polymers) varied with NaCl concentration, as shown in Fig. 2. With CApS the intensity decreases as salt is added, whereas with NApS the intensity increases slightly. Notably, the intensity curves with CApS and NApS converge to a common level at about 50 mM NaCl. For later use we note that this level corresponds to about 50% of the intensity with CApS at 5 mM NaCl, the ionic strength used in the flocculation studies [15].

In order to quantify the strength of the TNS binding to CApS, emission spectra at a constant concentration of TNS (0.5 ppm by weight) were recorded in the presence of various concentrations of CApS (0.025–0.2 %wt), at a constant ionic strength of 5 mM NaCl. Background scans of CApS without added TNS (but with the same emission/excitation filter settings) were subtracted from the corresponding TNS spectra to correct for polymer scattering. Figure 3a shows that the spectral shapes were insensitive to the mixing ratio (with a maximum at 460 nm) but that the intensity increases as the concentration of CApS increases. The increase is interpreted as reflecting how an increasingly larger fraction of TNS becomes bound to CApS as the concentration of binding sites increases. A plot of the intensity level at 460 nm versus amylopectin concentration (Fig. 3b) exhibits only a very weak curvature, however, and does not reach a saturation level even at the highest CApS concentrations. This shows that under the present conditions it was not possible to reach conditions where all TNS is bound. Attempts to increase the CApS concentration further led to polymer dissolution problems, and if the TNS concentration was decreased further the limit of detection sensitivity was surpassed. The limiting fluorescence intensity when all TNS is bound could thus not be measured, and the quantum yield of the bound form had to be kept as a proportionality parameter in the analysis of the binding constant.

The dissociation constant, K_d, of the CApS-TNS complex was obtained by assuming binding of TNS to a single type of site (S) on the amylopectin (see Discussion)

$$[\mathrm{TNS}\cdot\mathrm{S}] \Leftrightarrow [\mathrm{TNS}]_{free} + [\mathrm{S}]_{free} \quad , \tag{1}$$

where $[\mathrm{TNS}]_{free}$ is the concentration of nonbound TNS, $[\mathrm{S}]_{free}$ is the concentration of sites on the amylopectin which are not occupied by TNS and [TNS·S] is the concentration of bound TNS (and of occupied sites).

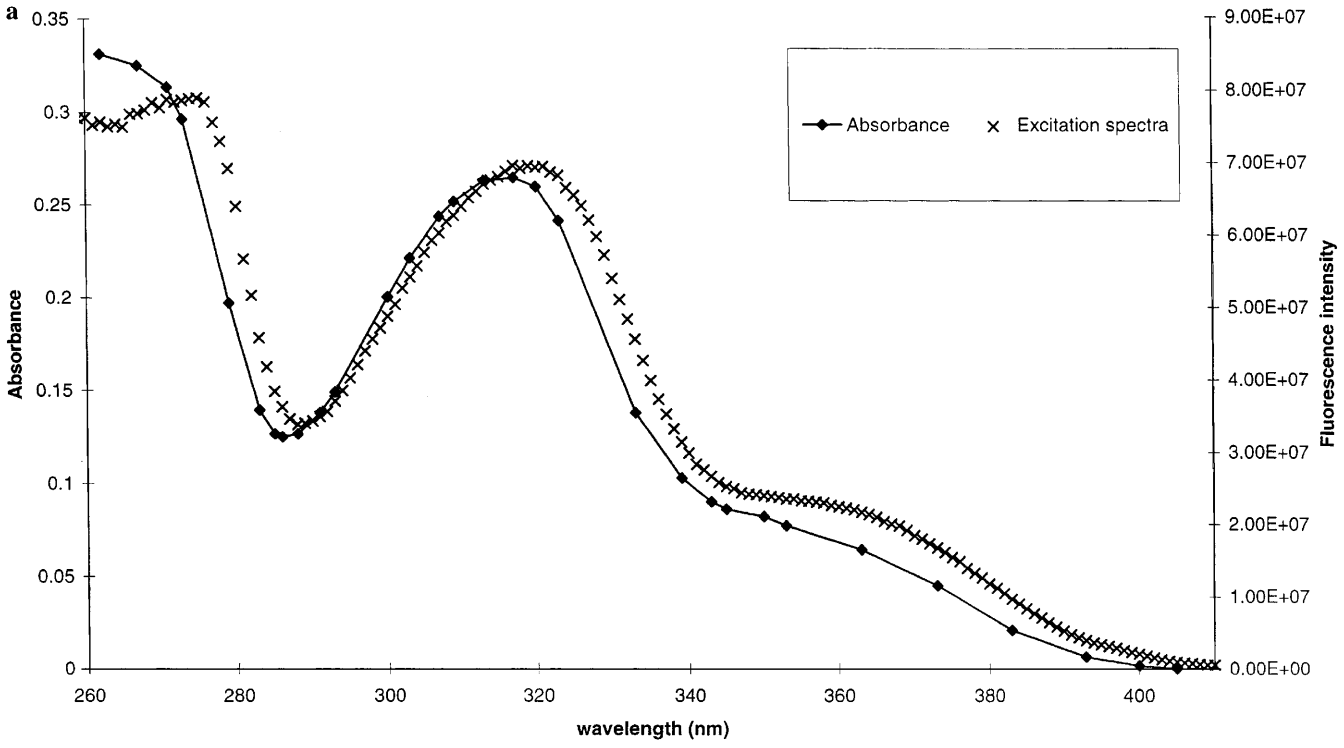
Then the dissociation constant K_d for the TNS-amylopectin complex is given by

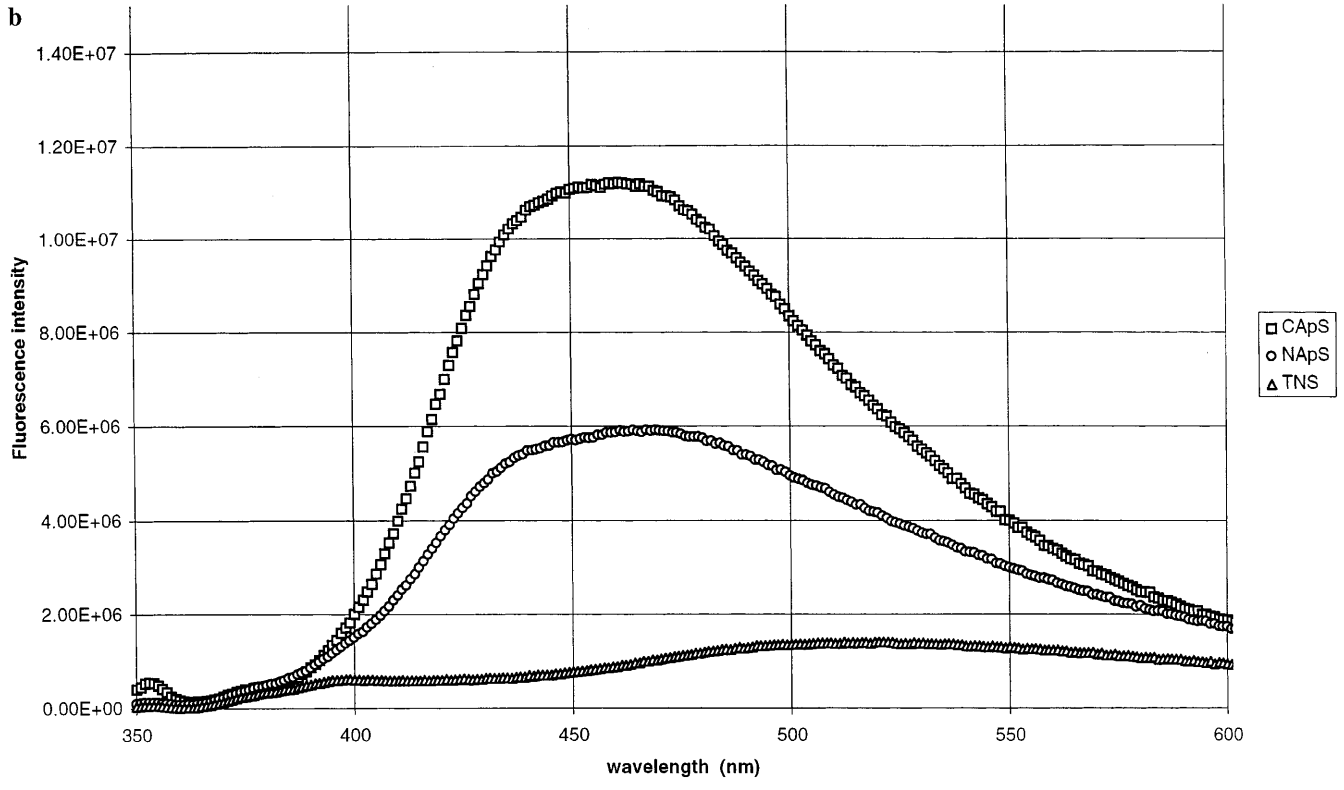
$$K_d = \frac{[\mathrm{TNS}]_{free}[\mathrm{S}]_{free}}{[\mathrm{TNS}\cdot\mathrm{S}]} \quad . \tag{2}$$

In order to facilitate a fit of the binding isotherm data in Fig. 3b, a linearized relation between the TNS fluorescence intensity and the amylopectin monomer concentration was derived from Eq. (2)

$$\frac{1}{f} = \frac{1}{nC[\mathrm{TNS}]_{tot}} + \frac{[\mathrm{TNS}]_{tot} + K_d}{nC[\mathrm{TNS}]_{tot}[\mathrm{glucose}]_{tot}} \quad , \tag{3}$$

where f is the fluorescence intensity at 460 nm, n is the size of each S in terms of the number of glucose monomers, C is a proportionality constant such that $f = C[\mathrm{TNS}]_{bound} = C[\mathrm{TNS}\cdot\mathrm{S}]$. Furthermore, $[\mathrm{glucose}]_{tot} = n\cdot([\mathrm{TNS}\cdot\mathrm{S}] + [\mathrm{S}]\mathrm{free})$ is the total concentration of amylopectin monomer and $[\mathrm{TNS}]_{tot} = [\mathrm{TNS}]_{free} + [\mathrm{TNS}\cdot\mathrm{S}]$ is the total concentration of TNS, both of which are known. A plot of the reciprocal fluorescence intensity at λ_{max} versus the reciprocal total amylopectin monomer concentration was linear. The fitted curve constants and the derived values of the binding parameters are given in Table 1. The constant C may be determined from an intensity measurement in the limiting case of a large excess of binding sites, but since this state could not be achieved only the product $C\cdot n$ could be determined.


a
Absorbance
Excitation spectra
Absorbance
Fluorescence intensity
wavelength (nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
9.00E+07
8.00E+07
7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
260
280
300
320
340
360
380
400


b
Fluorescence intensity
wavelength (nm)
CApS
NApS
TNS
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
350
400
450
500
550
600

◀

Fig. 1 a The absorbance and excitation spectra of 2-(*p*-toluidine)-6-naphthalene sulfonate (*TNS*) in the presence of cationic amylopectin potato starch (*CApS*). The concentration of TNS was 0.0005 %wt and the concentration of CApS was 0.05 %wt. The concentration of NaCl was 5 mM and the pH was 8. The emission wavelength was 460 nm. **b** The emission spectra of TNS in water, and in the presence of CApS and native amylopectin (*NApS*). The experimental conditions are as in **a**. The excitation wavelength was 315 nm. The peak around 350 nm is a water Raman peak

Discussion

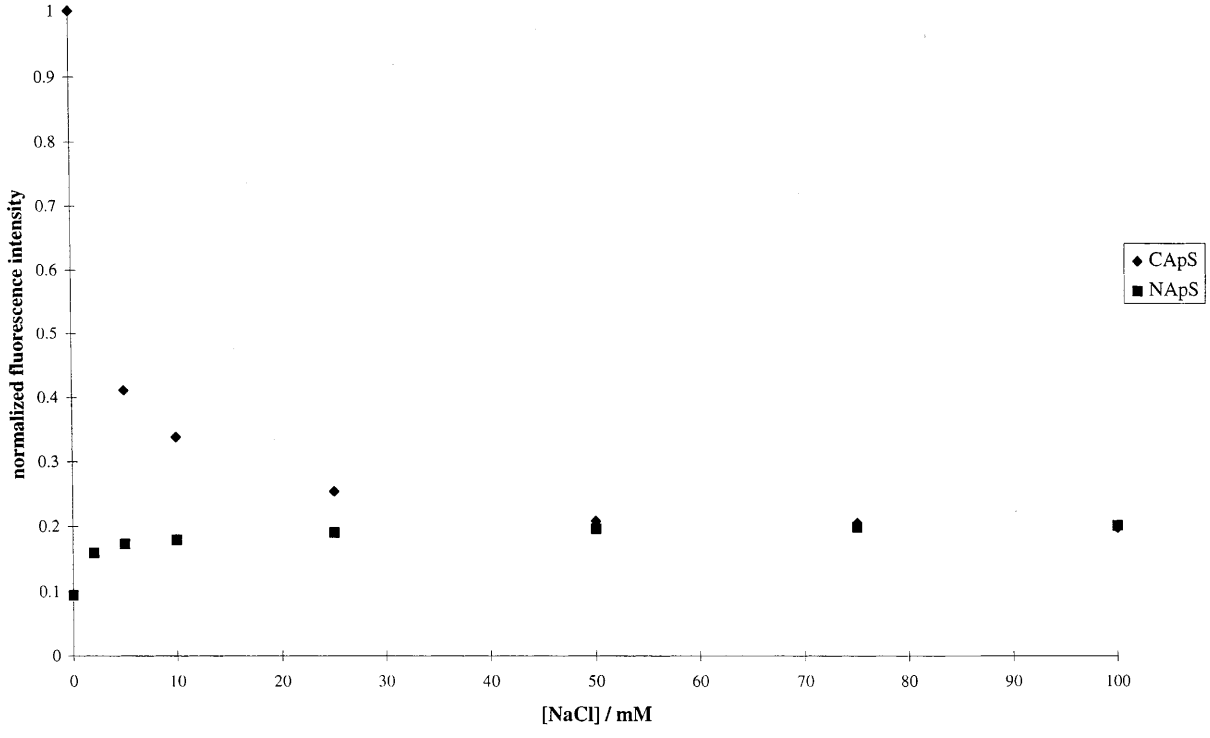
TNS binds to one type of binding site in amylopectin

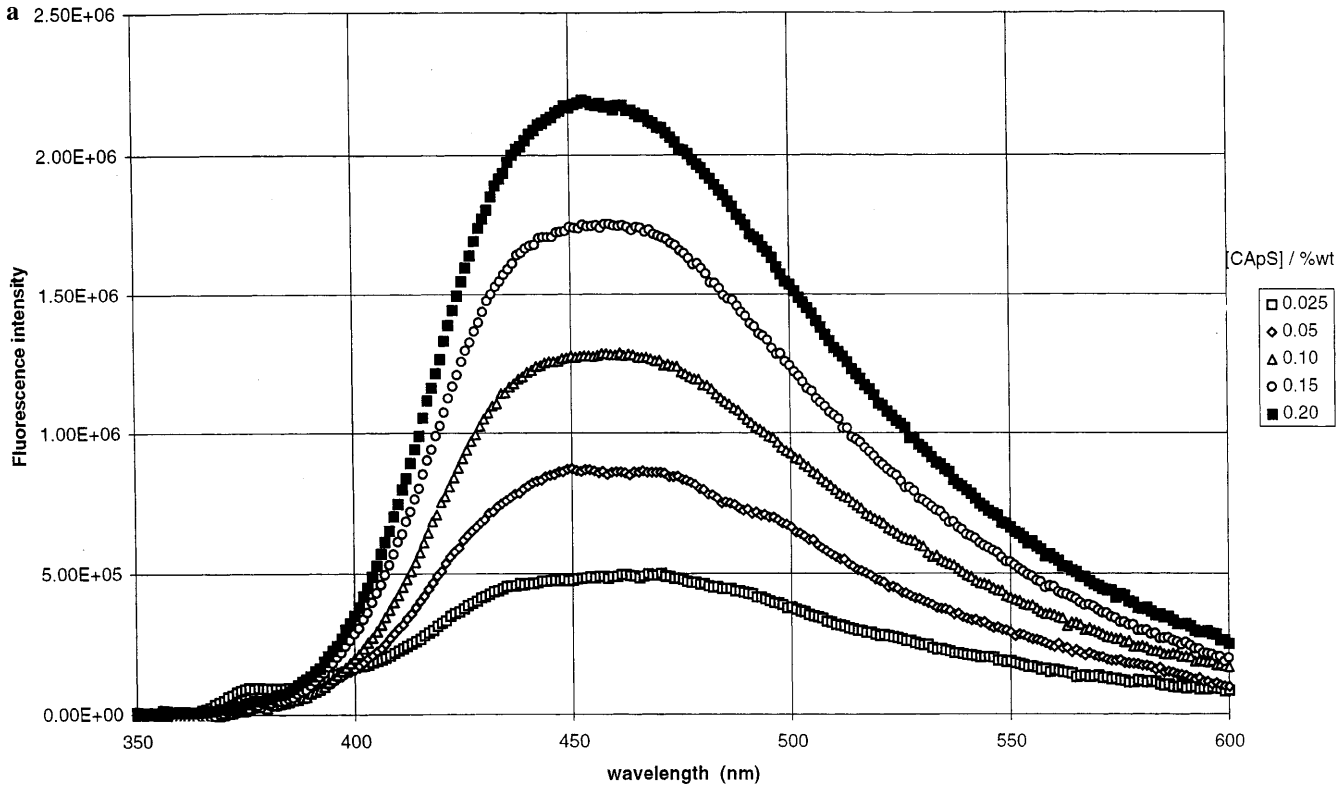
Since none of the two polymers are fluorescent in the wavelength range investigated, the observed changes in the fluorescence (Fig. 1b) show that TNS forms complexes with both polysaccharides NApS and CApS. Since the emission spectrum was independent of the excitation wavelength in both cases, evidencing a single emitting species, it can be concluded that TNS binds to one type of binding site in each of the polysaccharides. In principle a second emitting species is present, in the form of free TNS (because the binding is weak), but the quantum yield is so low compared to the bound form that in practice the latter dominates the emission spectra.

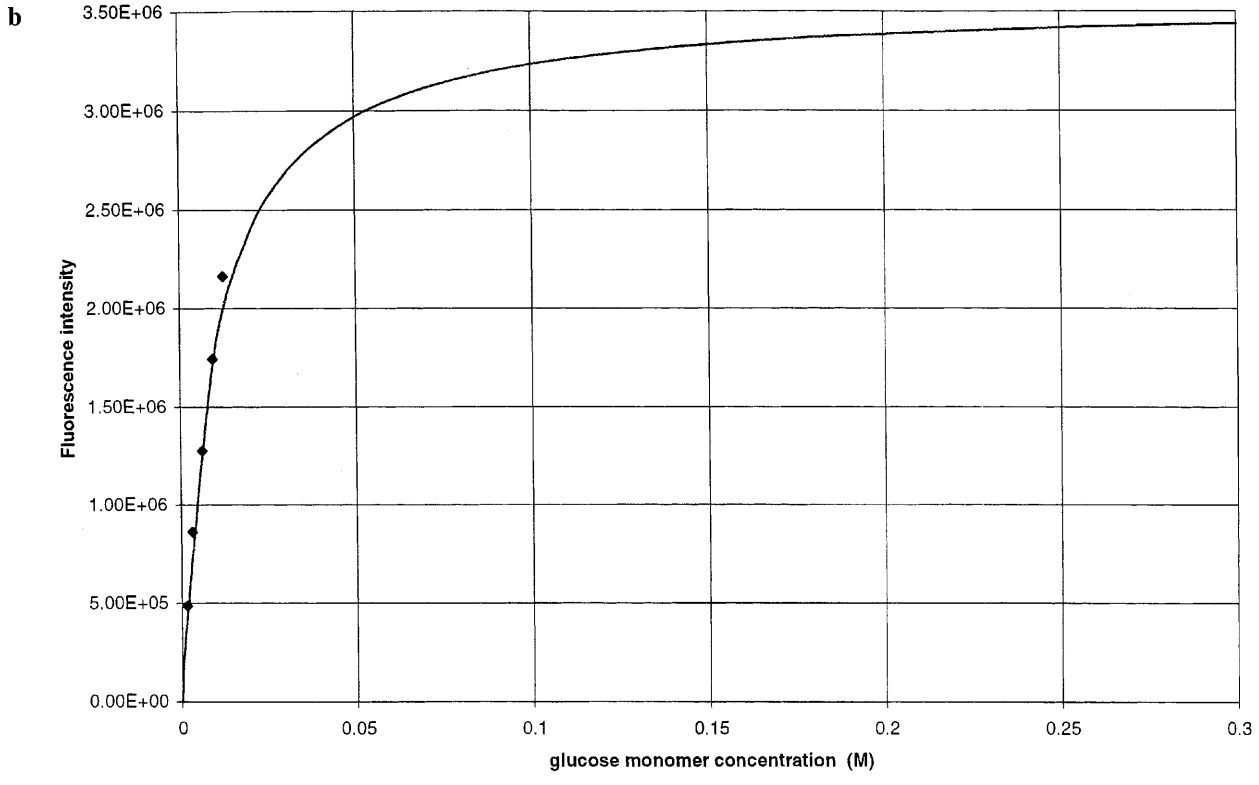
Fig. 2 Normalized fluorescence intensity at emission λ_{max} of TNS in the presence of CApS or NApS as a function of NaCl concentration. The concentration of TNS was 0.005 %wt. The concentration of carbohydrates was 0.05 %wt. The wavelength emission maximum was 460 nm for CApS and NApS

The binding site is of a nonpolar nature and is very similar in CApS and NApS

The fluorescence of anilino-naphthalenesulfonates (such as TNS) has been subject of numerous investigations [26–36]. In a detailed study Karukstis et al. [36] proposed that the fluorescence of TNS can be attributed to one of two types of excited states, depending on the polarity of the surrounding environment. In solvents of low polarity emission occurs from a π-π^* excited state. Polar solvents, on the other hand, stabilizes a charge-transfer state which occurs by intramolecular electron transfer from the phenyl ring to the naphthyl ring. The charge-transfer state has an emission maximum around 495 nm, whereas the π-π^* excited state has an emission maximum at about 450–460 nm and a considerably higher quantum yield of fluorescence [26] than the charge-transfer state. The enhancement and blueshift in emission to 460 nm when TNS binds to CApS (Fig. 1b) thus indicates that the binding site is less polar than water and that the emission is from the π-π^* excited state. Investigations of TNS physisorbed to various proteins have shown that the fluorescence of TNS bound to proteins with hydrophobic binding sites is stronger than the fluorescence in solutions of proteins without




a
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Fluorescence intensity
350
400
450
500
550
600
wavelength (nm)
[CApS] / %wt
0.025
0.05
0.10
0.15
0.20


b
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Fluorescence intensity
0
0.05
0.1
0.15
0.2
0.25
0.3
glucose monomer concentration (M)

◀

Fig. 3 **a** Emission spectra of TNS versus concentration of added CApS. The concentration of TNS was 0.00005 %wt. **b** Plot of maximum intensity (460 nm) in **a** versus CApS concentration. The CApS concentration is given in terms of glucose monomer concentration. The *line* corresponds to the best fit of Eq. (3)

such sites [27, 35]. The effect of hydrophobic pockets may be viewed as a dehydration that destabilizes the charge-transfer state. This mechanism would be quite general, and could account for the enhanced quantum yield upon interaction with CApS.

The very similar shape of the TNS spectrum when TNS is bound to NApS and CApS (Fig. 1b) strongly indicates that the (single) type of binding site is the same as in CApS. The generally lower emission intensity with NApS (Fig. 1b) may seem to contradict this; however, the binding to NApS will be weaker overall, due to the absence of electrostatic affinity. We therefore interpret the lower intensity as reflecting fewer bound molecules, rather than weaker intensity per bound dye. Strong support for this hypothesis, in addition to the similar spectral shapes, is the fact that the emission of TNS bound to CApS and NApS is of very similar intensity above 50 mM salt (Fig. 2), where the electrostatic part of the affinity for CApS can be expected to be screened to a large extent.

The effect of ionic strength on the binding affinity

Fundamental to our strategy of using TNS in flocculation studies is the assumption that the emission intensity reflects the amount of bound dye. The salt titration (Fig. 2) was conducted to test this hypothesis, and indeed revealed a decrease in intensity as expected from screening of the electrostatic attraction between anionic TNS and cationic CApS. In principle, the effect of ionic strength on TNS emission intensity could reflect direct salt effects on the dye itself, rather than on the affinity between dye and polymer. Salt had little effect on the emission of free TNS in water (results not shown), but since emission occurs from different electronic states for free and bound TNS this support for insignificant salt effects on TNS itself is not fully conclusive. However, the fact that the changes in emission with increasing salt with CApS and NApS are in opposite directions is difficult to renconcile with a direct effect of ionic strength on the quantum yield of TNS, since the TNS is bound to very similar types of sites in the two cases. Instead, the observation that the common high-salt fluorescence intensity level is reached from above with CApS but from below with NApS is fully consistent with screening of an attractive and a (weaker) repulsive electrostatic contribution, respectively, to the affinity for CApS and NApS. That the effect of salt is not on the optical properties of the dye itself, but rather indirectly on the amount of bound TNS is also supported by the retained spectral shape during the salt titration. The fact that the emission intensity reflects the fraction of TNS bound to the amylopectins was exploited to evaluate a dissociation constant of $K_d = 9 \pm 1$ mM for the TNS-CApS complex (Table 1), assuming the single-site model (Eq. 1) which is justified by the observation that there is only one type of binding site on the amylopectins.

Notably the release of TNS occurs over a considerably wider range of salt concentration (0–50 mM) than the range 0–2 mM where the majority of the decrease in coil size occurs [10, 12, 25]. It is therefore not likely that TNS is released as a direct consequence of a contraction of the amylopectin coils. The proposed screening mechanism is also supported by the observation that the electrophoretic mobility of CApS decreases by screening up to about 50 mM added NaCl [11].

In the limit of high ionic strength the TNS emission intensity with both CApS and NApS plateaus at a level which is significantly higher than for TNS in water. This shows that TNS remains bound to both polymers even when the electrostatic contributions are effectively screened. Since CApS and NApS give the similar high-salt intensity levels it is expected that the nonelectrostatic component is of similar strength in both polymers. An important observation is that the nonelectrostatic binding is fully reversible, even if the remaining TNS cannot be released by salt. This can be concluded from the observation [15] that anionic NSP can displace all the bound TNS. The nonelectrostatic attraction most likely involves hydrogen bonding since TNS contains a potential donor and amylose (the linear starch polymer) is known to form hydrogen bonds in other circumstances [25]. The strength of the interaction can be expressed more quantitatively by observing that the high-salt intensity (with CApS and NApS) is about 50% of the level observed with CApS at an ionic strength of 5 mM, whereas the intensity in water corresponds to only about 3% of that level. Thus, roughly speaking, the nonelectrostatic binding component corresponds to about 50% of the affinity for CApS at 5 mM salt. The dissociation constant of 9 ± 1 mM corresponds to an association constant of 110 ± 20 M^{-1}, which shows that the TNS-CApS complex is only weakly associated even at the

Table 1 Binding data for 2-(*p*-toluidine)-6-naphthalene sulfonate to cationized amylopectin potato starch at 5 mM NaCl

$\lambda_{excitation}$ (nm^{-1})	Slope (10^9)	Intercept (10^7)	$nC(10^{-11})$	K_d (mM^{-1})	r^2
350	7.86	9.25	7.60	8.50	0.997
315	2.74	2.81	0.250	9.75	0.997

[a] Slope and intercept of plot of data in Fig. 3b according to the linearized Eq. (3)
[b] Binding parameters calculated from the value of the slope and intercept using Eq. (3)

comparatively low ionic strength of 5 mM NaCl used in the flocculation work [15].

In summary, TNS binds reversibly to amylopectins at one type of site which has a significant nonelectrostatic contribution to the affinity in both CApS and NApS, with the major difference being that in CApS those sites offer an electrostatic attractive component, whereas in NApS there is a comparatively weak repulsive contribution. We also note that the ionic strength is an important parameter to be controlled in polyelectrolyte titrations with TNS as an indicator.

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