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Preparation and characterisation of fluorescent chitosans using 9-anthraldehyde as fluorophore

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Abstract

Chitosans with chemical composition ranging from a fraction of N-acetylated units (F_A) of 0.01 to 0.61 were used to prepare fluorescence labelled chitosans by reductive amination with 9-anthraldehyde. Fluorescent chitosans with a low theoretical degree of substitution (DS, 0.001–1%) were prepared, and the actual DS of the products were determined by UV and ^{1}H NMR spectroscopy. The fluorescence excitation and emission spectra of the chitosan with F_A of 0.09 and DS 1% showed an excitation maximum at 254 nm and an emission maximum at 413 nm. The intrinsic viscosities ([η]) of the fluorescent chitosans were compared to those of the original chitosans, showing that the derivatisation procedure lead only to a negligible decrease in [η]. The conformation of these fluorescent chitosans with very low DS-values is not altered and they can conveniently be directly quantified by UV or fluorescence spectroscopy.

Keywords: Fluorescent chitosans; Degree of substitution; Intrinsic viscosities

1. Introduction

Chitosan is a copolymer of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc, **A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN, **D**-unit) obtained by de-N-acetylation of chitin, a structural polysaccharide in the exoskeleton of arthrophods and the cell wall of certain fungi.[1] Chitosans and its derivatives are currently being studied for potential uses in such diverse fields as drug and gene delivery,[2–8] enzyme immobilisation,[9] polymer batteries,[10] antimicrobial effect,[11] and metal ion removal from aqueous solutions.[12]

Quantitative analysis of polysaccharides implies total hydrolysis of the monosaccharide(s). However, highly de-N-acetylated chitosans are relatively resistant to acid hydrolysis due to their positively charged 2-amino groups.[13] Enzymes such as chitosanases may be used for depolymerisation of chitosans,[14] however both availability and cost limit the use of enzymes. Chitosans can be quantified by the use of anionic dyes,[15] or a radioactive label.

We have previously reported on the use of ninhydrin for quantitative analysis of chitosan, finding that both the chemical composition and the chain length of the chitosans were

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important in order to obtain reliable calibration curves[16]. However, other primary amines (e.g. proteins) can also react with ninhydrin, excluding the use of the ninhydrin method to quantify chitosans in e.g. food systems.

In this paper we describe a simple method to introduce a certain amount of fluorescent groups into chitosans to make them fluorescent without essentially affecting their polymer properties. These labelled fluorescent chitosans may be used as standard samples for the quantitative analysis of chitosans by either fluorescence or UV spectroscopy. Fluorescence labelling of chitosan can be used as an alternative to radioactive assays with the additional advantage that the chitosans can be observed directly with e.g. fluorescence microscopy.[17] Another example is the use in ultracentrifugation studies of chitosans.[18] Fluorescence labelled chitosans may also be useful to detect chitosans in biological systems.

2. Experimental

Materials.—The following chitosans with different intrinsic viscosities ($[\eta]$) and fractions of N-acetylated units (F_A), were provided by Pronova Biopolymer (Drammen, Norway) and used as starting material (**1**): No. 1 ($[\eta] = 1100$ mL/g, $F_A = 0.15$), No. 2 ($[\eta] = 230$ mL/g, $F_A = 0.09$), No. 3 ($[\eta] = 900$ mL/g, $F_A = 0.61$) and No. 4 ($[\eta] = 830$ mL/g, $F_A = 0.01$). All final products were purified by dialysis using Spectra/por 3 (molecular weight cut-off 3500 Da). The fluorophore 9-anthraldehyde (**2**) was purchased from Aldrich. A mixture of **2** (varying amounts) and sodium cyanoborohydride (157 mg, 2.5 mmol) in MeOH (20 mL) was stirred at rt over night. The mixture was evaporated to one third of the original volume, and then water (10 mL) was added. The precipitated product was collected and recrystallised (1:1 MeOH–water) as light yellow needles, confirmed by ^{1}H NMR and melting point to be 9-anthracenemethanol (ANM), also available commercially.

Analysis.—The fluorescence analysis was performed on a Perkin–Elmer LS-5B luminescence spectrometer. The fluorescent excitation and emission scan spectra of fluorescent chitosans (**3**) with different degrees of substitution (DS) were acquired from 2.0×10^{-6} M (GlcN/GlcNAc units) water solutions using a 10-mm cuvette. The fluorescent emission scan spectra of **2** and ANM were acquired from 2.0×10^{-8} M MeOH solutions. The ^{1}H NMR spectrum of **3** was acquired by a Bruker DPX 400 spectrometer at 400.13 MHz and 90 °C (10 mg in 0.7 mL D_2O). All samples were supplied with 5 μL of a 1% solution of sodium 3-(trimethylsilyl)propionate-d_4 in D_2O as an internal standard.[19] In order to ensure that the relative areas of the observed resonances represent the relative amounts of protons involved, especially for the protons in the 9-anthryl group relative to the chitosan protons, we accumulated free induction decays with a 30° pulse and a repetition time of 13 s. Spectra with high signal-to-noise ratios were recorded by obtaining 256 scans of each sample. UV spectra were recorded on a Perkin–Elmer UV–Vis spectrophotometer on solutions of **2**, ANM (2.0×10^{-6} M in MeOH) and fluorescent chitosan (2.0×10^{-4} M GlcN/GlcNAc units in water). The calibration curve was obtained by measuring the absorbance of increasing concentrations of ANM ($0.50–11 \times 10^{-6}$ M) at 250 nm. Determination of DS for the different fluorescent chitosans was also performed at this wavelength. Intrinsic viscosities were measured at 20 °C by the use of an Ubbelohde Viscosimeter (Type Schott 53101/0a) as previously described.[20]

The coupling reaction.—To a solution of chitosan **1** (1.0 g, 2–5 mmol GlcN) in 2% AcOH (120 mL), a solution of **2** (in the chosen amount) in MeOH (120 mL) was added. The mixture was stirred for 24 h at rt, sodium cyanoborohydride (4.5 mg, 0.07 mmol) added and the stirring was continued for 12 h. The reaction mixture was evaporated under vacuum at a temperature below 30 °C until the remaining volume was approximately 100 mL, and then extracted with EtOAc (3×100 mL) to remove excess reagents. The water phase was dialysed first against 0.2 M NaCl and then against deionised water. The pH of the dialysed solution was adjusted to 4.5 with HCl before lyophilisation. White fluorescent chi-

tosan-chlorides with DS of 0.001–1% were obtained in yields generally exceeding 90%. The data of the starting chitosans and the fluorescent chitosans are listed in Table 1.

3. Results and discussion

9-Anthraldehyde (**2**) was introduced on the different chitosans by reductive amination as shown in Scheme 1. The condensation reaction of a primary amino group (nucleophile) with a carbonyl group (an aldehyde or a ketone) results in an unstable imino bond by the elimination of a water molecule. This Schiff base type of equilibrium reaction is strongly dependent on the pH of the solution and other factors stabilising or destabilising the product, such as water concentration, degree of conjugation of the resulting product,[21] and the

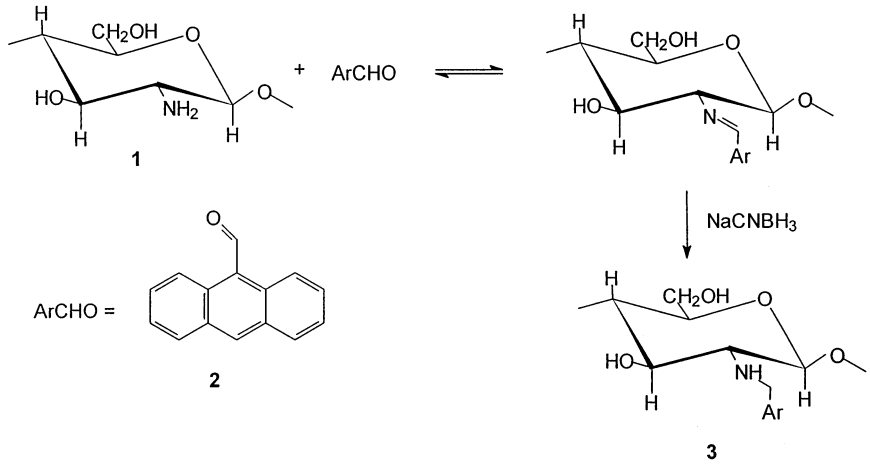
Table 1
Theoretical and actual degree of substitution (DS), yield, intrinsic viscosity ([η]) and measured log ε for the fluorescent chitosans

Starting chitosan	Fluorescent chitosan				
	DS [a] (mol%)	DS [b] (mol%)	Yield (%)	[η] (mL/g)	Log ε (254 nm)
Chitosan No. 1	0.014	0.02	98	950	1.34
$F_A = 0.15$, [η] = 1100 mL/g	0.14	0.14	97	810	2.18
	1.4	1.2 [1.0]	97	1030	3.11
Chitosan No. 2	0.0014	0.008	98	230	0.92
$F_A = 0.09$, [η] = 230 mL/g	0.014	0.06	96	240	1.79
	0.14	0.26	95	170	2.45
	1.4	2.7 [1.0]	96	210	3.47
Chitosan No. 3	0.014	0.02	97	850	1.26
$F_A = 0.61$, [η] = 900 mL/g	0.14	0.12	98	810	2.12
	1.4	1.6 [0.9]	96	700	3.24
Chitosan No. 4	0.014	0.04	97	850	1.67
$F_A = 0.01$, [η] = 830 mL/g	0.14	0.25	96	850	2.44
	1.4	1.6	96	840	3.24

ε: the extinction coefficient from the Lambert–Beer law: $A = \varepsilon cl$. DS: degree of substitution (mol% of sugar units (GlcNAc + GlcN) substituted with **2**).

[a] Theoretical value.

[b] Value calculated from UV measurements using calibration curve (Fig. 4). Values in brackets are determined by ^{1}H NMR spectroscopy.



Scheme 1. Reductive amination of chitosan (**1**) with 9-anthraldehyde (**2**) in the presence of $NaCNBH_3$ to obtain the fluorescent chitosan (**3**).

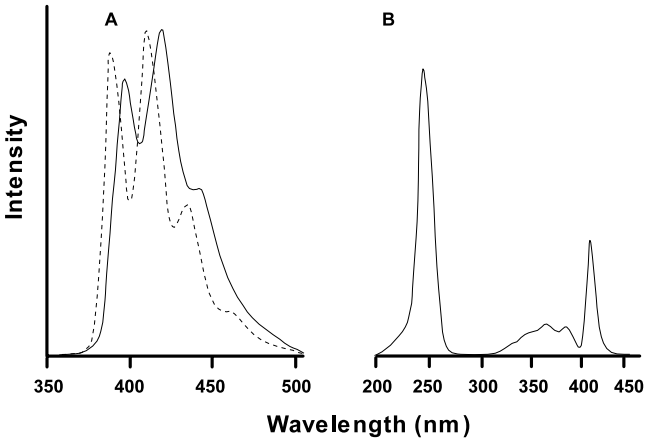


Fig. 1. (A) Fluorescent emission scan spectra obtained by excitation at 254 nm. The reductive amination product **3** with DS 1% ($F_A = 0.09$) in water (—) and 9-anthracenemethanol (ANM) in methanol (---). (B) Fluorescent excitation scan spectrum of product **3** with DS 1% ($F_A = 0.09$) in water.

choice of sodium cyanoborohydride ($NaCNBH_3$) as the reducing compound has the advantage that it selectively reduces the imino bonds at pH > 4.[21] In this case, the amino groups of chitosan (**1**) is the nucleophile condensing with the aldehyde group of 9-anthraldehyde (**2**), resulting in the secondary amine of the fluorescent chitosan (**3**).

The molar ratios of monomers of **1**:**2** in the coupling reaction were ~100:1, 1000:1, 10,000:1 and 100,000:1, in order to synthesise chitosans with a chromophore which can conveniently be visualised and quantified by UV or fluorescence spectroscopy, without altering the conformation of the polysaccharide. The fluorescent chitosans were characterised, and the data are shown in Table 1. Although a similar reaction has been reported previously,[22] the properties and spectroscopic data of the product were not given. In the following discussion, we have chosen the modified chitosan No. 2 (see Table 1) with DS 1% as an arbitrary example. The fluorescent properties of **3** were compared with 9-anthracenemethanol (ANM).

The emission scan spectrum of **3** in water (excitation wavelength 254 nm) is shown in

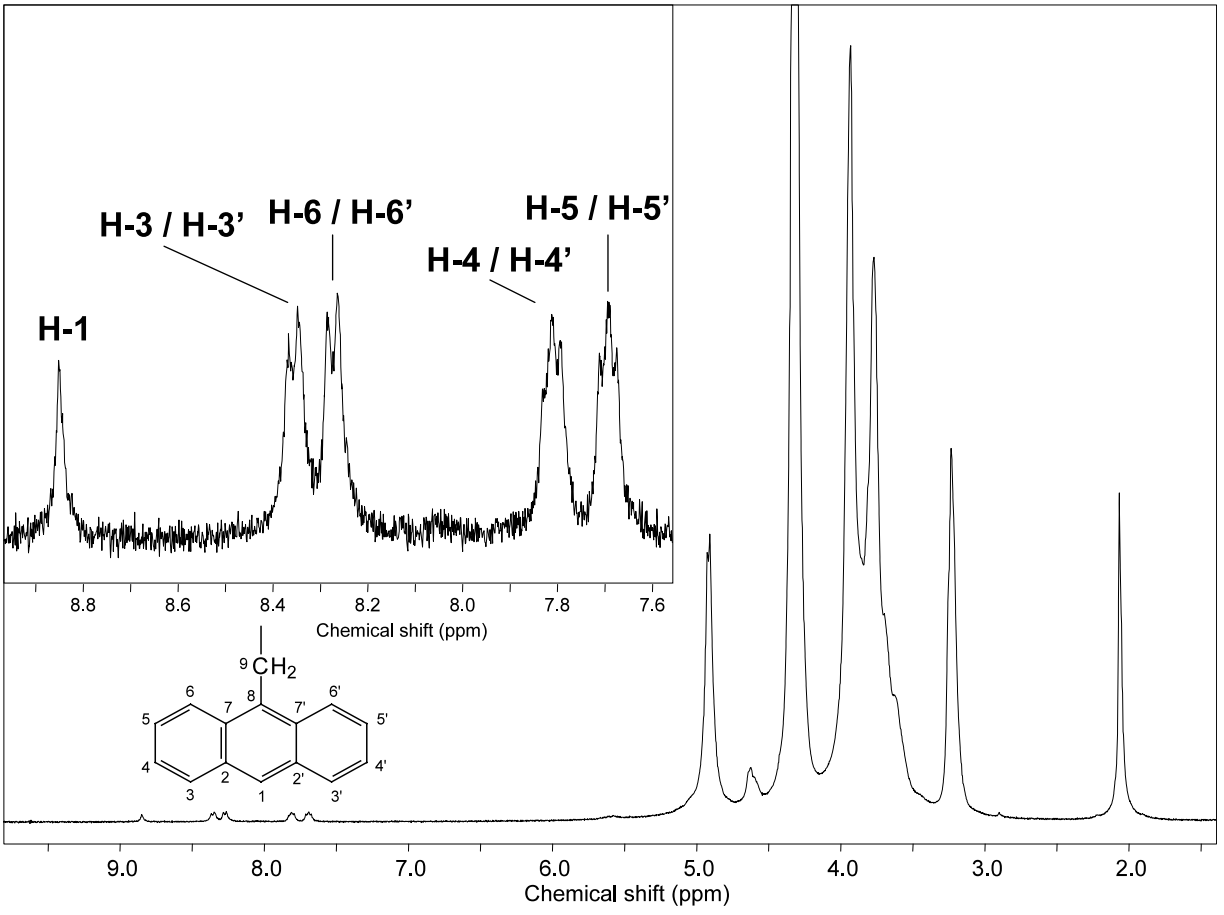


Fig. 2. ^{1}H NMR spectrum of the fluorescent chitosan **3**, where the aromatic region is enlarged (DS 1%, $F_A = 0.15$, 400 MHz, D_2O, pD 4).

Fig. 1(A, solid line) and this is similar to that of ANM in methanol (dashed line), but has shifted 5 nm towards longer wavelengths. The two starting materials **1** and **2** have no emission in this region. The maximum emission wavelength of 254 nm was found in the excitation spectrum of **3** (Fig. 1(B)). Fluorescent chitosans with different DS show the same emission and excitation scan spectra (data not shown). No significant difference was observed between the maximum emission wavelengths (Em_{max}) of different chitosan samples.

In the ^1H NMR spectrum of **3** (Fig. 2), the signals at δ 2.05 (MeCO–), 3.15 (H-2, **D**-unit), 3.5–4.0 (H-3, H-4, H-5 and H-6) and 4.85 (H-1, **D**-unit) and 4.60 (H-1, **A**-unit) showed the characteristic protons of chitosan. The assignment was made by direct comparison to reported data.[23,24] The five small resonances in the aromatic region (7.65–8.85 ppm) were assigned to the protons in the 9-anthryl group as indicated in Fig. 2. The signals for the two-methylene protons (–CH_2N–) were not assigned, and probably overlap with the chitosan resonances.

The intrinsic viscosities ([η]) of the fluorescent chitosans were only marginally lower than those of the starting chitosans (Table 1). It is therefore concluded that the chitosans can be derivatised by this procedure without significant degradation. The fluorescent chitosans were prepared with affirmed low substitutions, making it unlikely that the fluorescent chromophore should affect the conformation and other properties of the polysaccharide, as also concluded from conformation studies using analytical ultracentrifugation.[18] The UV spectrum of the modified chitosans **3** (Fig. 3(a)) is similar to that of the reference ANM (Fig. 3(b)), but different from that of the 9-anthraldehyde (**2**) (Fig. 3(c)). Underivatised chitosan has no UV adsorption in this region (data not shown). The λ_{max} of **3** (254 nm) is very close to that of ANM (250 nm). This makes it possible to determine the DS of **3** by using ANM as a standard reference, assuming that the extinction coefficients of **3** and ANM are similar. Two methods have been used for the determination of the DS of **3**. Firstly, the integrals of the aromatic proton signals and that of the signals from H-1 of GlcNAc (4.60 ppm) and GlcN (4.85 ppm) in the proton NMR spectrum (Fig. 2) were used to determine the DS. The aromatic resonances were too weak for those chitosans with DS lower

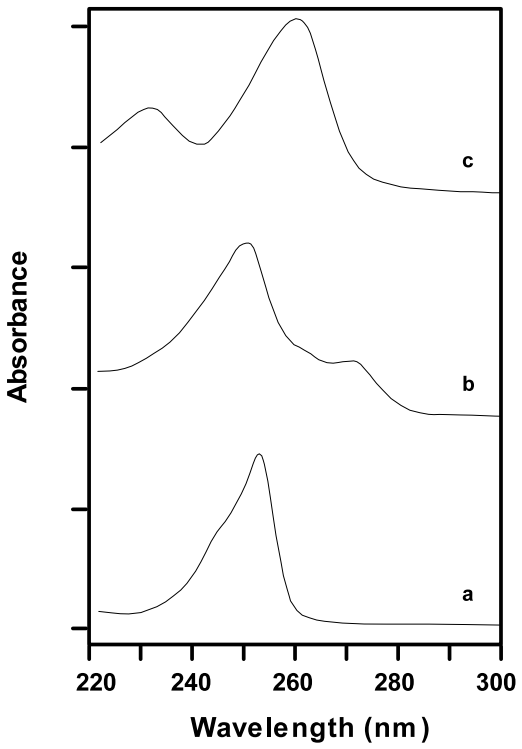


Fig. 3. UV spectra of (a) fluorescent chitosan **3** (DS 1%, $F_A = 0.09$) in water; (b) 9-anthracenemethanol (ANM) in methanol; (c) 9-anthraldehyde (**2**) in methanol.

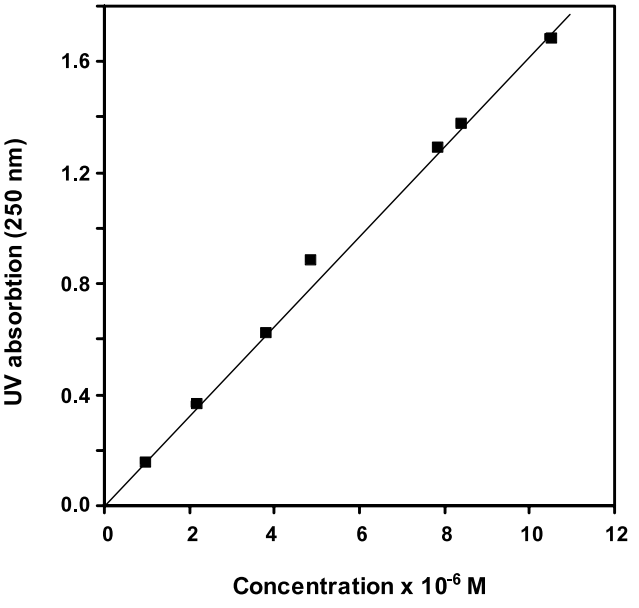


Fig. 4. Calibration curve for quantitative determination of DS values for fluorescent chitosans by UV spectroscopy, prepared by measuring the absorbance of increasing concentrations of 9-anthracenemethanol (ANM) in methanol.

than 1% to provide a reliable estimation of the DS. Secondly, UV spectroscopy was used. The UV method is based on the assumption that the 9-anthryl group is the main chromophore in the modified chitosans and the reference, and that this chromophore determines the UV absorption in both **3** and ANM. These two compounds have similar UV curves and the correlation of the absorbance to the concentration of the fluorescent chitosan **3** is also linear. This can be used to obtain a calibration curve to determine the concentration of the 9-anthrylmethylated unit in the modified chitosan **3**, as shown in Fig. 4.

4. Conclusions

The condensation of chitosans with 9-anthraldehyde, i.e., Schiff base formation, followed by reduction with sodium cyanoborohydride renders an easy way to obtain fluorescent chitosans that emit at Em_{max} 413 nm when excited at Ex_{max} 254 nm. The degree of substitution of the obtained fluorescent chitosan **3** will depend on the molar ratio of the two starting materials, and can be quantitatively determined by UV or NMR spectroscopy. The fluorescent chitosans can be prepared without significant depolymerisation of the polysaccharide. Like chitosan, polymer **3** was water soluble at acidic pH values, and insoluble in methanol and ethanol. Incorporation of the fluorophore label will allow both detection and quantification of chitosan in different applications.

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