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Precise derivatization of structurally distinct chitosans with rhodamine B isothiocyanate

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Abstract

Work to date shows that structurally distinct chitosans have reacted inefficiently and unpredictably with fluorescein isothiocyanate (FITC) in an acid–methanol solvent that maintains both chitosan and fluorophore solubility. Since isothiocyanate preferentially reacts with neutral amine groups, and chitosan solubility typically depends upon a minimal degree of protonation, we tested the hypothesis that precise derivatization of chitosan with rhodamine isothiocyanate (RITC) can be achieved by controlling the reaction time and the degree of protonation. Addition of 50% v/v methanol reduced the chitosan degree of protonation in acetic acid but not HCl solutions. At various degrees of protonation, chitosan reacted inefficiently with RITC as previously observed with FITC. Nevertheless, precise derivatization was achieved by allowing the reaction to proceed overnight at a given degree of protonation ($p < 0.0001$, $n = 18$) and fixed initial fluorophore concentration. A reproducible 2% to 4% fraction of neutral amines reacted with RITC in proportion to the initial fluorophore concentration ($p < 0.005$). Using our optimized protocol, chitosans with different degree of deacetylation and molecular weight were derivatized to either 1% or 0.5% mol/mol RITC/chitosan-monomer with a precision of 0.1% mol/mol. The average molecular weight of fluorescent RITC-chitosan was similar to the unlabeled parent chitosan. Precise molar derivatization of structurally distinct chitosans with RITC can be achieved by controlling chitosan degree of protonation, initial fluorophore concentration, and reaction time.

Keywords: Chitosan; Fluorescence; Degree of deacetylation; Molecular weight; Rhodamine B isothiocyanate; Rhodamine B; Extinction coefficient; Degree of protonation

1. Introduction

Chitosan is a biocompatible polysaccharide employed in medical applications where the rate of cell uptake and clearance is of paramount importance. Chitosan structure can vary in terms of molecular weight and molar percent glucosamine [glucosamine/(glucosamine + *N*-acetyl glucosamine)] which is also known as the degree of deacetylation (DDA). Fluorescent chitosans derivatized with fluorescein isothiocyanate (FITC) have been previously used to show that chitosan DDA and molecular weight can influence cell binding, *in vivo* absorption and *in vivo* clearance (Chae, Jang, & Nah, 2005; de Campos, Diebold, Carvalho, Sanchez, & Alonso, 2004; Huang, Khor, & Lim, 2004; Onishi & Machida, 1999). To pin-point structural features that affect chitosan-cell interactions, specifically in regenerative medicine applications involving transient in situ chitosan

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residency (Buschmann, Hoemann, Hurtig, & Shive, 2006; Hoemann et al., 2005; Shive et al., 2006), the derivatization level using any given fluorophore should be held constant when comparing structurally distinct chitosans. Using this principle, and a library of equally derivatized FITC-chitosans, Chae et al. (2005) determined that increasing chitosan molecular weight suppresses *in vivo* intestinal absorption, although the authors of this study did not provide a detailed description of the labeling procedure in DMSO.

A procedure for coupling chitosan with FITC in acid–methanol has been described (Huang, Ma, Khor, & Lim, 2002; Qaqish & Amiji, 1999). However we determined that this method has previously yielded widely varying and uncontrolled 24–91% coupling efficiencies between FITC and chitosan (Table 1). We therefore aimed to optimize the acid–methanol labeling procedure to produce structurally distinct chitosans precisely derivatized with 1.0% mol/mol rhodamine B isothiocyanate (RITC). Our strategy intended on the one hand to minimally alter chitosan DDA and on the other hand to have acid-stable fluorescent derivatives for comparative intracellular tracking in live cells. We hypothesized that the reaction could be best controlled by holding constant the time, temperature, and solution pH. Given that isothiocyanate optimally reacts with neutral amine groups at pH 9.0, we considered that solution pH was a most critical variable because homogenous labeling requires chitosan to be fully soluble, while most chitosans are insoluble when a substantial number of amine groups are neutral at pH $\geqslant$ 6.5 (Nordtveit, Varum, & Smidsrod, 1994; Rinaudo, Pavlov, & Desbrieres, 1999a). Using a labeling protocol optimized for reaction time and chitosan degree of protonation, we determined the effect of performing the chitosan labeling reaction in dilute acetic acid versus dilute HCl with chitosans of variable deacetylation levels and different molecular weights generated either by nitrous acid degradation or hydrochloric acid hydrolysis (Fig. 1).

2. Methods

2.1. Materials

Medical-grade free-base chitosans with defined DDA and molecular weight (Table 2) were provided by BioSyntech (Laval, QC, Canada) and certified to contain <0.2% w/w protein, <500 EU/g endotoxin, and <10 ppm heavy metals. All chitosans were either lyophilized or weighed taking into account water content (loss on drying). Rhodamine B isothiocyanate ($C_{29}H_{30}ClN_3O_3S\cdots Cl^-$, 536 g/mol, mixed isomers, Product No. R1755), rhodamine B

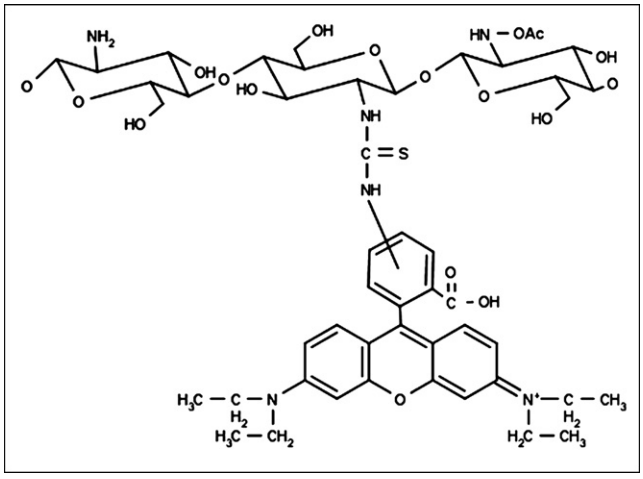

Fig. 1. Rhodamine isothiocyanate-chitosan.

Table 1
Derivatization levels previously reported for the reaction of chitosan with FITC were used to determine the labeling efficiency

Author	Chitosan %DDA, M_W	Labeling solvent	Reaction time (h)	FITC/chitosan-monomer % mol/mol[a]		Labeling efficiency (%)
				Initial	Final	
Qaqish & Amiji, 1999	85	AcOH–methanol[b]	1	2.0	1.3	65
Onishi & Machida, 1999	50	water pH 6.9[c]	24	3.3	3.0	91
Huang et al., 2002	90.2, 180 K	AcOH–methanol[b]	3	4.2	1.1	26
Huang et al., 2004	46, 213 K	AcOH–methanol[b]	3	4.7	2.7	57
Huang et al., 2004	88, 213 K	AcOH–methanol[b]	3	4.2	1.0	24
Huang et al., 2004	88, 10 K	AcOH–methanol[b]	3	4.2	3.6	86
Tikhonov, Lopez-Llorca, Salinas, & Monfort, 2004	85	AcOH–methanol[d]	12	>1.9[e]	2.2	–
Chae et al., 2005	88, 3.8 K	H_2O DMSO	24	0.40	0.38	95
Chae et al., 2005	92, 13 K	H_2O DMSO	24	0.40	0.38	95
Chae et al., 2005	85, 230 K	H_2O DMSO	24	0.40	0.35	88

Abbreviations: FITC, fluorescein isothiocyanate; AcOH, acetic acid; K, kilodaltons.

[a] Molar derivatization levels were based on FITC M_W 389.4 g/mol and chitosan-monomer (glucosamine + *N*-acetyl glucosamine) M_W average unit = (1 − DDA) × 203 g/mol + (DDA) × 162 g/mol; 50% DDA = 182 g/mol, 85% DDA = 167 g/mol, 92% DDA = 163 g/mol.

[b] 50 mM acetic acid/50% v/v methanol.

[c] 50–70% DDA chitosan has been shown to be soluble at neutral pH (Varum, Ottoy, & Smidsrod, 1994).

[d] 80 mM acetic acid/50% methanol.

[e] Insolubles removed prior to labeling.

Table 2
Structural characterization of chitosans used in this study

Lot No. (sample name)	DDA (%)	M_n (PDI)[a](kDa), (M_W/M_n)	M_n (PDI)[b](kDa), (M_W/M_n)	Dynamic viscosity (mPa s)
Chitosan degree of protonation in acid-methanol				
86	81.7	–	–	448[e]
61	94.6	243 (1.4)	248 (1.5)	845[e]
Pilot labeling study				
64	82.6	–	–	568[e]
75	80.2	166 (1.5)	161 (1.6)	26[e]
Labeling kinetics				
23	71.7	–	220 (1.5)[c]	3355[e]
75	80.2	166 (1.5)	161 (1.6)	26[e]
09	98.4	–	120 (1.5)[c]	136[e]
Targeted labeling				
23 (M39D71.7)	71.7	–	39 (4.0)[c]	–
23 (M185D71.7)	71.7	–	185 (2.3)[c]	–
57 (M38D80)	80.0	–	38 (2.6)[c]	–
57 (M153D80)	80.0	–	153 (1.6)[c]	–
60 (M38D92.7)	92.7	–	38 (1.6)[c]	–
60 (M151D92.7)	92.7	–	151 (1.4)[c]	–
75 (80L)	80.2	183 (1.2)	149 (1.6)[d]	178[f]
92 (80M)	80.6	176 (1.4)	179 (1.5)[d]	1422[f]
61 (95L)	94.6	79 (1.5)	78 (1.5)[d]	197[f]
61 (95M)	94.6	147 (1.6)	168 (1.5)[d]	2964[f]

Abbreviations: DDA, degree of deacetylation; M_n: number-average molecular weight; M_W: weight-average molecular weight; PDI polydispersity index (M_W/M_n); L for low and M for medium viscosity.

[a] M_n and M_W determined with column sets guard-PW_{XL}-G6000 PW_{XL}-G5000PW_{XL}.

[b] M_n and M_W, determined with a linear column $GMPW_{XL}$.

[c] Nitrous acid degraded, M_n and M_W from Lavertu, Methot, Tran-Khanh, and Buschmann (2006).

[d] Autoclave acid-heat degraded at pH 5.6.

[e] 1% w/w solution in 0.1 N HCl.

[f] Post-autoclave viscosity for 2% w/w solution in HCl pH 5.6.

($C_{28}H_{31}ClN_2O_3$, 479 g/mol, Product No. 25-242-5), and 1 N HCl (tissue culture grade) were from Sigma–Aldrich (Oakville, ON, Canada). Anhydrous methanol (Product No. VW5790-0) and glacial acetic acid (99.7%, Product No. 9515-03), acetic acid 1 N concentration (Product No. VW3236-1), and 0.45 μm pore size syringe filters were from VWR (Montreal, QC, Canada) or Millipore (Mississauga, ON, Canada).

2.2. Chitosan degree of protonation in the labeling solvent

A Neslab RT-111 instrument with temperature-controlled water bath equipped with a Ross epoxy body electrode (Model 8115BNUWP, Sigma-Aldrich, Oakville ON, Canada) pH electrode and temperature probe was used to measure the pH and temperature of solutions which were kept in a 50 mL beaker. The electrode was calibrated using pH standards in water without methanol. The effect of acid concentration, using a strong or weak acid, on chitosan solution pH in water versus 50% v/v methanol/water was examined using chitosan at two different DDA levels. The glucosamine concentration (mol/L) was calculated as DDA × (chitosan concentration, g/L)/$\bar{M}_W$ where $\bar{M}_W$ is the average monomeric molecular weight that is a function of DDA (Table 3).

$$\bar{M}_W = \text{DDA} \times 161\ \text{g/mol} + (1 - \text{DDA}) \times 203\ \text{g/mol} \quad (1)$$

Chitosan was dissolved in either HCl or acetic acid at a 0.7 (81.7% DDA) or 0.75 (94.6% DDA) molar ratio of [acid]/[glucosamine], to obtain 30 ml of 50 mM glucosamine (1.03% w/v and 0.863% w/v, respectively). The solution was combined with an equal volume of either water or methanol. Chitosan was fully soluble at these acid concentrations with or without 50% methanol. The pH was recorded before and after each step during step-wise addition of either 0.100 N HCl or 0.100 N acetic acid in increments of 0.05 [acid]/[glucosamine] at 25 °C. The average pH of duplicate samples which differed by less than 0.05 pH unit was used to calculate the pK_a and degree of

Table 3
Examples of glucosamine and acid concentrations in a 20 mL labeling reaction mixture prior to adding 50% v/v methanol, and mass of fluorophore added after addition of methanol

DDA	$\bar{M}_W$ chitosan average monomer unit (g/mol)	[Monomer] at 1% w/v (mM)	[Glucosamine] at 1% w/v (mM)	[Acetic acid] at 0.7 molar ratio [AcOH]/[gluc][a] (mM)	rhoB-ITC at 2% mol/mol[b] (mg)
Chitosan degree of protonation in acid-methanol					
0.817	169	59	49	35	–
0.946	163	61	58	41	–
Chitosan labeling					
0.72	173	58	42	29	12.9
0.77	171	59	45	32	13.1
0.80	169	59	47	33	13.2
0.826	168	59	49	35	13.3
0.93	164	61	57	40	13.6
0.95	163	61	58	41	13.6
0.98	162	62	61	42	13.8

Abbreviations: DDA, degree of deacetylation; $\bar{M}_W$, average monomeric molecular weight.

[a] For a 1% w/w chitosan solution prior to adding an equal volume of methanol.

[b] The dry mass of rhodamine B isothiocyanate (536 g/mol, 96% w/w when corrected for chloride content) was adjusted to obtain 2.0% mol/mol rhoB-ITC/chitosan according to Table 2, with an accuracy of ± 0.4 mg.

protonation of chitosan as described in the next section below.

2.3. Chitosan degree of protonation in the labeling solvent

The degree of protonation (α) is the molar ratio of protonated glucosamine to total glucosamine. For chitosan solutions dissolved in HCl, with or without methanol, α is equal to [HCl]/[glucosamine] due to electroneutrality since Cl^- concentration must equal protonated glucosamine when H^+ is negligible. In order to calculate α in the solution of acetic acid and 50% v/v anhydrous methanol, the chitosan pK_a in this solvent is required and therefore determined by dissolving desiccated 81.7% DDA or 94.6% DDA chitosan in water at 50 mM glucosamine using an [HCl]/[glucosamine] molar ratio of 0.75 and adding an equal volume of anhydrous methanol as described in the preceding section. This solution was then titrated with 0.100 N HCl as described above and the apparent dissociation constant pK_a as a function of α was determined in the titrated range $0.75 < \alpha < 0.90$, according to

$$pK_a(\alpha) = pH(\alpha) + \log_{10}\frac{\alpha}{1-\alpha} \quad (2)$$

using α = [HCl]/[glucosamine]. In order to calculate α in acetic acid/methanol we assumed the chitosan pK_a to be independent of the counterion type (Cl^- or CH_3COO^-) and therefore used the chitosan pK_a values determined in HCl/methanol solvent. However we needed to extrapolate pK_a values obtained for chitosan/HCl to values of α that were lower than the range measured in HCl/methanol ($0.75 \leqslant \alpha \leqslant 0.90$). This was due to the observed increase in pH in acetic acid/methanol that was up to 0.5 pH units higher than the highest pH measured in HCl/methanol (i.e. pH measured at [HCl]/[glucosamine] = 0.75). The linear extrapolation had the form (Filion, Lavertu, & Buschmann, 2007):

$$pK_a(\alpha) = -m\alpha + b \quad (3)$$

where m and b ($b = pK_0$, the intrinsic dissociation constant of the polymer) are positive parameters that were obtained by fitting pK_a versus α for the HCl/methanol titration. The degree of protonation (α) of chitosan in acetic acid/methanol was then calculated by combining Eqs. (2) and (3) to yield:

$$\alpha = \frac{1}{1+10^{(pH-pK_a(\alpha))}} = \frac{1}{1+10^{(pH+m\alpha-b)}} \quad (4)$$

It should be noted that the above approach was taken rather than using the pK_a of acetic acid and the electroneutrality condition to determine α, since the pK_a of acetic acid in solutions containing chitosan and methanol has not yet been precisely determined.

2.4. Chitosan degradation to obtain polymers with distinct DDA and molecular weight

A library of six nitrous acid-degraded chitosans with three DDA levels and high or low molecular weight (Table 1) were previously generated and characterized in Lavertu et al. (2006). A second library of four chitosans was generated by autoclave-HCl hydrolysis. Solutions consisting of 2% w/v chitosan, 80% DDA or 95% DDA in dilute HCl (α = 0.8, pH 5.6), were autoclaved for 30–150 min yielding chitosans of medium (M) (1000–1500 mPa s) or low (L) (150–300 mPa s) viscosity (Table 1). Chitosan solution dynamic viscosity was determined at 25 °C, at 10–100 rpm with a Brookfield viscometer (LVDVI+, Brookfield Engineering Laboratories Inc. Middleboro, MA), and a spindle chamber SC4-14/6R with 2 mL sample volume. Autoclave-sterilized solutions were diluted to 1% w/v chitosan with sterile deionized water prior to labeling with RITC.

2.5. Chitosan labeling with rhodamine B isothiocyanate

Endotoxin contamination of labeled chitosan was minimized by the use of sterile solutions and glassware. For a typical labeling experiment, 200 mg chitosan was dissolved overnight at 1% w/v chitosan in acetic acid with a total volume of 20 mL in a foil-covered erlenmeyer flask. The acetic

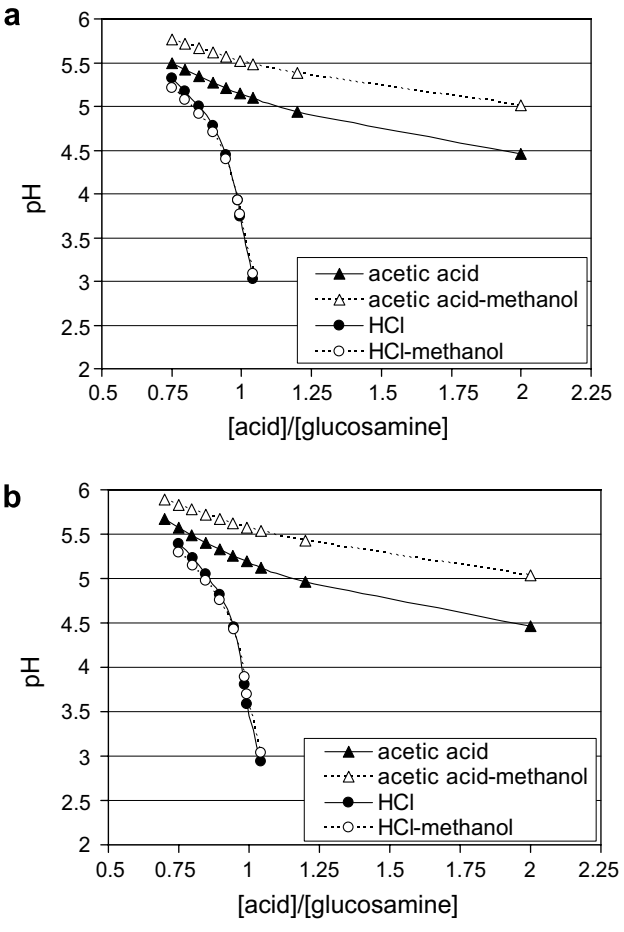

Fig. 2. Solution pH of chitosans as a function of the [acid]/[glucosamine] molar ratio in water and in 50% v/v methanol at 25 °C. (a) 94.6% DDA chitosan. (b) 81.7% DDA chitosan.

acid concentration corresponded to either 100 mM (as in Qaqish & Amiji, 1999), to 60 mM, or to 0.7 molar ratio [acetic acid]/[glucosamine] (see Table 3). An equal volume of anhydrous methanol was added to the chitosan solution, followed by stir bar agitation for 3 h. The flask was sealed and nitrogen gas bubbled into the solution for 15 min to remove oxygen. To initiate the reaction with 2% mol/mol RITC/chitosan-monomer, 12.9–13.8 mg of RITC (taking into account chloride salt mass, and depending on the chitosan DDA, see Table 3) was dissolved at 2 mg/mL in anhydrous methanol to ensure full RITC solubility before adding drop-wise to the chitosan solution with constant agitation. The final concentration of methanol was 56% v/v methanol/acid. The reaction was maintained at room temperature (19° to 22 °C) by placing a thin slab of styrofoam between the stir plate and the reaction vessel to prevent heat transfer from the stir plate apparatus. The reaction was allowed to proceed for 18 h in the dark. For kinetic labeling experiments, aliquots were drawn at intervals up to 72 h from a reaction as described above, of 350 mg chitosan dissolved at 0.7 molar ratio [acetic acid]/[glucosamine] and either 3.5% mol/mol (80.2% DDA) or 2% mol/mol (71.7% or 98.4% DDA) RITC/chitosan-monomer. Structurally distinct chitosans were labeled with 2% mol/mol RITC/chitosan-monomer for 18 h at 0.7 molar ratio [acetic acid]/[glucosamine] ($\alpha = 0.5$, nitrous acid-degraded chitosans), or 17 h at 0.8 molar ratio [HCl]/[glucosamine] ($\alpha = 0.8$, HCl-degraded chitosan solutions).

At the end of the labeling period, RITC-chitosans were precipitated by adding 0.2–0.5 mL 10 N NaOH, and repeatedly washed with deionized water until the wash solution was neutral with $OD_{556} < 0.01$, and freeze-dried. An average 10% loss of RITC-chitosan mass occurred during alkaline precipitation and washing to eliminate free fluorophore. Labeling efficiency was determined by the OD_{556} of a 0.1 mg/mL RITC-chitosan solution dissolved at a molar ratio [HCl]/[glucosamine] = 0.9, according to the Beer–Lambert Law ($OD_{556} = \varepsilon \cdot c \cdot l$), and Eq. (5).

$$\%\text{mol/mol} = \frac{\left[\mathrm{OD}_{556} \Big/ (1\text{cm} \times 87{,}000(\text{cm} \cdot \text{mol/L})^{-1})\right]}{0.1\text{g/L}/\bar{M}_{\mathrm{W}}\ \text{g/mol}} \times 100\% \tag{5}$$

The extinction coefficient for rhodamine B $\varepsilon = 87{,}000$ (cm mol/L)$^{-1}$ was obtained from the OD_{556} of serial dilutions in water or Tris pH 6.8 that gave similar values and where the $\bar{M}_{\mathrm{W}}$ of chitosan was obtained from Eq. (1).

To determine the influence of pH, acid conjugate, and salt on filter sterile RITC-chitosan fluorescence, RITC-chitosan was dissolved at 5 mg/mL in water at $\alpha = 0.9$ (HCl), $\alpha = 1.2$ (HCl), or $\alpha = 1.0$ (acetic acid) and passed through a 0.45 μm filter. Solutions were further diluted to 50 μg/mL in deionized water with 0, 50 or 150 mM NaCl. RITC-chitosan solution fluorescence was determined by measuring 200 μL sample volumes in triplicate at 550 nm excitation, 580 nm emission, in black FluoroNunc 96-well plates (Fisher Scientific, Ottawa, ON, Canada) using a SpectraMax Gemini XS fluorimeter plate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

2.6. Chitosan molecular weight determination

Number-average molecular weight (M_{n}) and polydispersity index (PDI) of RITC-chitosan and unlabeled chitosan were determined using size exclusion chromatography (SEC). The SEC system was equipped with a multi-angle laser light scattering (MALLS) DAWN EOS (Enhanced Optical System) detector coupled with a differential refractometer Optilab DSP (Digital Signal Processor), both from

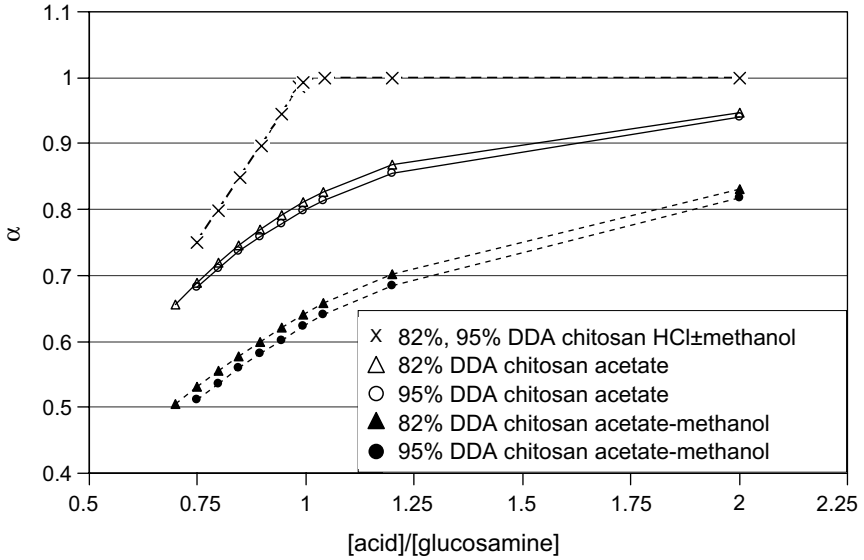

Fig. 3. Calculated chitosan degree of protonation (α) for 94.6% DDA and 81.7% DDA chitosan dissolved in acetic acid or HCl at distinct molar ratios of [acid]/[glucosamine], with and without 50% v/v methanol.

Wyatt Technology Corporation (Santa Barbara, CA, USA), and functioning at the wavelength 690 nm. The DAWN EOS was normalized with pullulan P-5 standard, (Shodex, Kawasaki, Japan). Free-base chitosan was dissolved overnight at 1.0 mg/mL in the SEC mobile phase buffer (0.3 M acetic acid, 0.2 M sodium acetate, 0.8 mM sodium azide, pH 4.5) (Brugnerotto, Desbrières, Roberts, & Rinaudo, 2001; Rinaudo, Milas, & Le Dung, 1993) and passed through a 0.45 μm syringe filter. Analyses were performed using column sets (TSKgel PW_{XL} series, Tosoh Bioscience, Montgomeryville, PA, USA), of either guard-PW_{XL}-G4000PW_{XL}-G3000PW_{XL} for <50 kDa molecular weight chitosans or guard-PW_{XL}-G6000PW_{XL}-G5000PW_{XL} for >50 kDa molecular weight chitosans. Data were presented as the average of duplicate measurements obtained from one sample (nitrous acid-degraded chitosans) or the range of duplicate measurements obtained from duplicate samples (acid-hydrolyzed chitosans). Autoclave-HCl hydrolyzed chitosan samples were also analyzed in duplicate by directly diluting chitosan-HCl into the GPC mobile phase buffer followed by SEC with Triple Detection with a Viscogel GMPW_{XL} linear column (Cat No 08025), a Viscotek 270 dual detector for low and right angle light scattering and viscosity (Viscotek Co., Houston TX) and refractive index detector (Agilent Technologies Product No. G1362A).

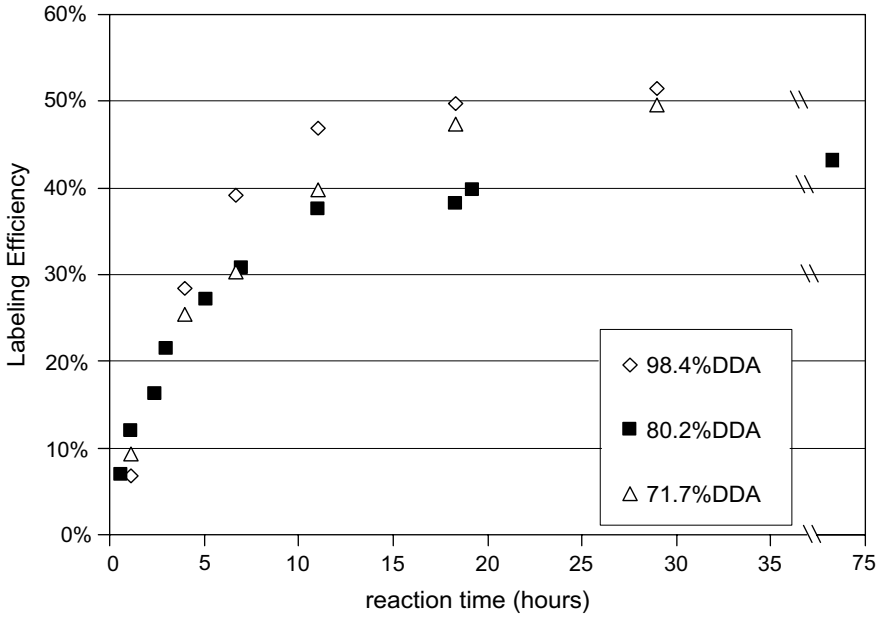

Fig. 4. Effect of reaction time on labeling efficiency of chitosan at three distinct DDA levels with RITC in acetic acid–methanol, chitosan $\alpha = 0.5$. Labeling efficiency indicates the percent of initially added fluorophore that is covalently attached to the chitosan.

Table 4
Characterization of RITC-chitosan libraries

Rho chitosan	Labeling efficiency (%)	% mol/mol rhoB/chitosan monomer	Pre-label		Post-label	
			M_n (KDa)	PDI (M_W/M_n)	M_n (KDa)	PDI (M_W/M_n)
Nitrous acid degraded[a]						
M39D71.7	44	0.9	39[a]	4.0	59[b]	1.4
M185D71.7	50	1.0	185[a]	2.3	68[c]	1.2
M38D80	46	0.9	38[a]	2.6	60[b]	1.3
M153D80	46	0.9	153[a]	1.6	129[c]	1.1
M38D92.7	49	1.0	38[a]	1.6	78[b]	1.1
M151D92.7	44	0.9	151[a]	1.4	172[c]	1.1
Acid-heat degraded						
80L	24	0.5	18–24,181–215[d]	2.0	81–134[d]	1.5
80M	24	0.5	175–177[d]	1.4	138–150[d]	1.3
95L	26	0.6	78–81[d]	1.5	84–119[d]	1.2
95M	25	0.6	133–161[d]	1.6	145–209[d]	1.1

Abbreviations: M_n: number-average molecular weight; M_W: weight-average molecular weight; PDI (M_W/M_n): polydispersity index.
[a] From Lavertu et al. (2006).
[b] Single sample analyzed, determined with column sets guard PW_{XL}-G4000 PW_{XL}-G3000PW_{XL}.
[c] One sample or [d]two distinct samples analyzed (range) with column sets guard PW_{XL}-G6000 PW_{XL}-G5000PW_{XL}.

2.7. Statistics

Multivariate correlation analysis using Statistica version 6.0 (StatSoft Inc., Tulsa, OK) was used to evaluate the relationship between the degree of protonation (α) and molar derivatization level. The Student t test was used to evaluate the influence of DDA and initial fluorophore concentration on percent neutral amine groups conjugated with fluorophore. Significance was considered for $p < 0.05$.

3. Results

3.1. Chitosan degree of protonation in acid–methanol

To optimize the reaction pH, we determined the chitosan degree of protonation in acid–methanol at specific [acid]/[glucosamine] ratios. Chitosans dissolved in acetic acid had a higher solution pH and a therefore a lower degree of protonation compared to chitosans dissolved in HCl at the same concentration (Figs. 2 and 3), consistent with previous literature (Rinaudo et al., 1999a). Methanol had a striking effect on chitosan dissolved in acetic acid, namely an increase in pH and a remarkable 15% drop in the chitosan degree of protonation (α) (Figs. 2 and 3). This methanol-dependent increase in solution pH was attributed to a methanol-dependent increase in the pK_a of acetic acid, from ~4.5 to ~5.2 although exact values were difficult to determine since chitosan will also influence the acetic acid pK_a. Chitosan neutralization (deprotonation) in the presence of 50% v/v methanol was specific to chitosan dissolved in acetic acid solutions and did not occur in HCl solutions where simple electroneutrality controls chitosan degree of protonation with or without methanol (Fig. 2). According to our data, chitosan was close to $\alpha = 0.8$ in the labeling solvent that is most frequently used to conjugate FITC to chitosan (de Campos et al., 2004; Huang et al., 2002, 2004; Qaqish & Amiji, 1999), that is 50 mM acetic acid, 50% v/v methanol, where the molar ratio of [acid]/[glucosamine] is 1.7–2.1 depending on the DDA (Fig. 3). By comparison, in a reaction medium with [acetic acid]/[chitosan] at 0.7 and 50% v/v methanol, the degree of protonation was reduced to $\alpha = 0.5$ (Fig. 3) which is approximately the minimal degree of protonation required for chitosan solubility in water (Rinaudo, Pavlov, & Desbrieres, 1999b).

3.2. Precise derivatization

Experiments were conducted to determine the effect of degree of protonation and reaction time on derivatization level while holding the reaction temperature constant at room temperature. After 4 h of reaction, the labeling efficiency increased from 11% to 19% (average of $n = 2$) by lowering the degree of protonation from $\alpha = 0.8$ to $\alpha = 0.5$, respectively. We next determined that 18 h of reaction was required for chitosans at three different DDA levels to become maximally coupled with RITC, consuming roughly half of the initially added fluorophore (Fig. 4). Around ~50% of the fluorophore remained in the labeling solvent (data not shown) and reproducibly failed to react with chitosan in acetic acid–methanol, pH 5.6.

To test whether the same derivatization level could be obtained independently of chitosan structure, two libraries

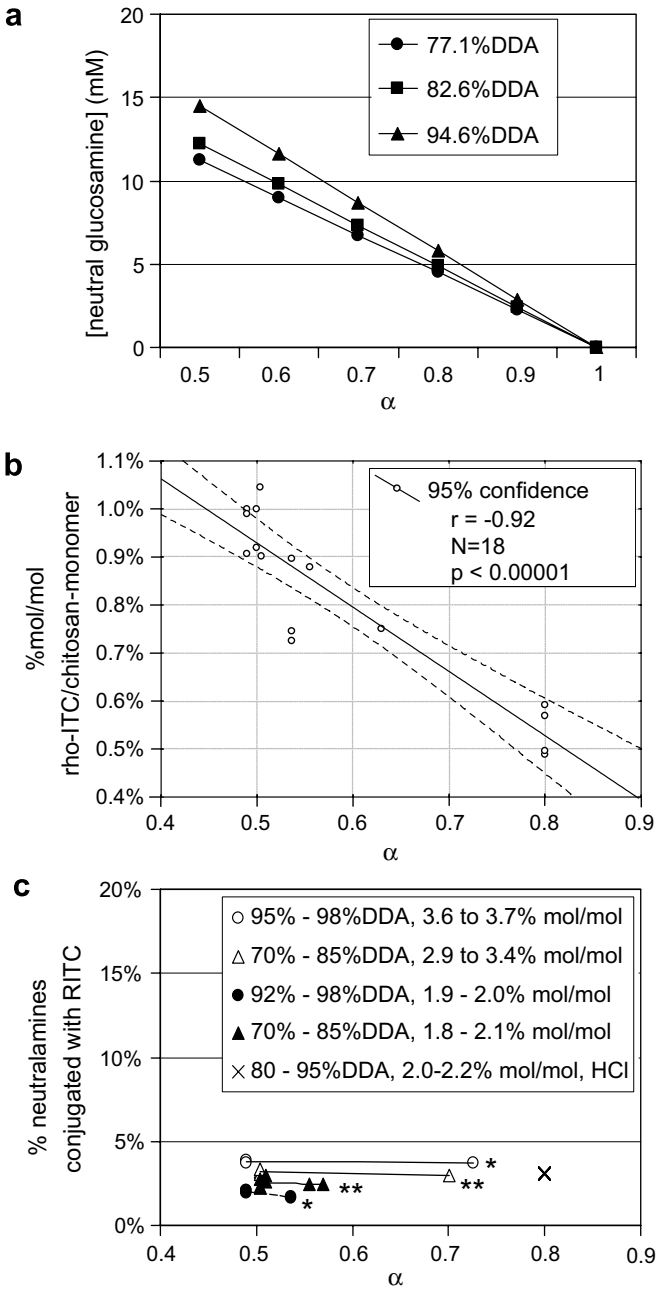

Fig. 5. (a) Theoretical concentration of neutral amines for 0.5% w/v chitosan at three different DDA levels in the reaction media (50% v/v methanol/acid) with variable degree of protonation, according to [glucosamine] * (1 − α). Note that the glucosamine concentration for 0.5% w/v chitosan is 50% of those concentrations given in Table 3 for 1% w/v solutions. (b) Chitosan molar derivatization (% mol/mol RITC/chitosan-monomer) was significantly correlated to degree of protonation (α). (c) The same fraction of neutral amines reacted with RITC irrespective of α. The fraction of reacted amines was influenced appreciably by the percent input fluorophore (*, **: $p < 0.005$, $n = 3$–7) and only slightly by the percent DDA.

of structurally distinct chitosans were labeled at a fixed degree of protonation. Six nitrous acid-degraded chitosans at $\alpha = 0.5$ were derivatized to 0.9–1.0% mol/mol RITC/chitosan-monomer, while four HCl acid-heat degraded chitosans at $\alpha = 0.8$ were derivatized to 0.5–0.6% mol/mol RITC/chitosan-monomer (Table 4). This reproducible ~2-fold higher derivatization at $\alpha = 0.5$ relative to $\alpha = 0.8$ coincided with a ~2.5-fold increase in the theoretical concentration of neutral glucosamine available to react at each respective degree of protonation (Fig. 5a). A comprehensive analysis of 18 labeling reactions carried out with 2% mol/mol RITC/chitosan-monomer revealed that derivatization level correlated with the α in the reaction medium ($p < 0.00001$, Fig 5b and Table 4). The derivatization level never attained the theoretical maximum (2% mol/mol) corresponding to complete consumption of RITC. However, the fraction of neutral amine groups reacted with RITC was constant over a range of α, and proportional to the initial concentration of fluorophore ($p < 0.005$, Fig. 5c).

The molecular weight of the chitosans before and after derivatization was measured by SEC multi-angle laser light scattering. We noted that the derivatized chitosans had significantly lower polydispersity indexes compared to the starting material (Table 4). The sharpening of the molar mass distributions may be in consequence of the purification protocol for labeled chitosans, or partial degradation of the main polymer chain. After derivatization, the molecular weight of all RITC-chitosans was within 50 kDa of the starting molecular weight (Table 4). Some variability in molecular weight measurements for 72–80% DDA chitosans (Tables 2 and 4) could also be explained by the known tendency for acetylated chitosans to form aggregates through hydrophobic interactions (Brugnerotto et al., 2001; Rinaudo et al., 1993).

3.3. Relative fluorescence of sterilized RITC-chitosans

To be useful for *in vivo* or cell culture applications, RITC-chitosans need to demonstrate stable fluorescent properties after sterilization. Samples subjected to autoclave sterilization completely lost their fluorescence, and those washed in their powder form with ethanol became totally resistant to re-solubilization. In contrast, filter-sterilization of 5 mg/mL solutions had a negligible effect on the relative fluorescence units (RFU). We tested the influence of acid conjugate, salt concentration and pH on RITC-chitosan fluorescence, specifically in a pH range that might be encountered in acid endosomes of live cells. Relative fluorescence of two structurally distinct RITC-chitosans, 80.2% or 94.6% DDA, derivatized with 1.0% mol/mol RITC was indistinguishable at pH 4.0–5.1 in salt-free solutions (Fig. 6). Moreover, salt had a similar quenching effect on relative fluorescence of 80.2% and 94.6% DDA RITC-chitosans (Fig. 6). These data demonstrated that similarly derivatized RITC-chitosans have highly comparable fluorescent properties. Relative fluorescence of all derivatives was proportional to the derivatization levels obtained by absorption at OD_{556} (Table 4).

4. Conclusion

Reaction conditions were established that permit precise coupling of rhodamine B isothiocyanate with structurally distinct chitosans. Precise labeling (0.5% mol/mol or 1.0% mol/mol) was achieved by generating chitosan solutions at specific degree of protonation in the reaction media ($\alpha = 0.8$ or $\alpha = 0.5$, respectively), by adding 2% mol/mol (6.6% w/w) fluorophore, and by allowing the reaction to proceed for 18 h. Using this protocol, other isothiocyanate-reactive compounds could also be conjugated to chitosan at targeted levels, including more expensive fluorophores such as Oregon green-isothiocyanate, as well as other molecules such as biotin-isothiocyanate.

The reaction conditions optimized in this study thus offer an improvement over previous FITC-chitosan labeling procedures for which, as a rule, derivatization was incomplete and unpredictable (Table 1). A reaction time of about 18 h was required for maximum fluorophore

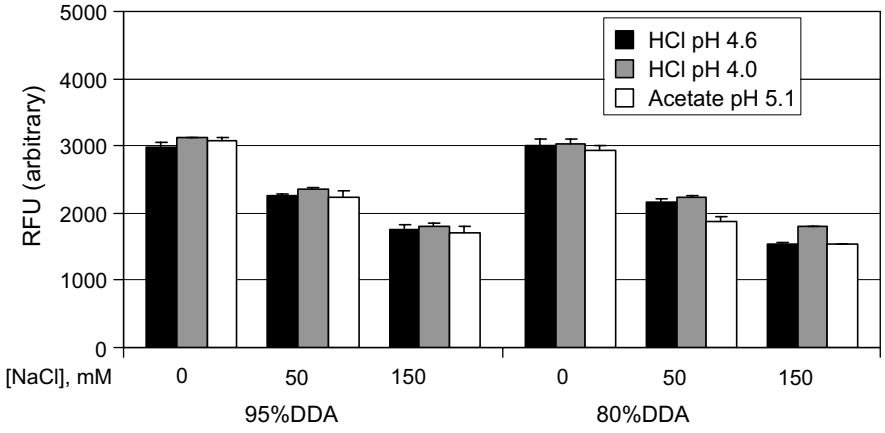

Fig. 6. Highly comparable fluorescent properties were observed for two structurally distinct RITC-chitosans with the same 1.0% mol/mol derivatization level, in different acids and salt concentrations, pH 4.0–5.1. RFU, relative fluorescence units. Mean ± SD ($n = 3$).

coupling to occur using standard experimental conditions. These data could explain the inconsistent FITC-chitosan derivatization levels previously obtained by Huang et al. (2004) for structurally distinct chitosans, since the reaction was terminated in this previous study after only 3 h. Our data support a model whereby the final derivatization level can be predicted based on the concentration of neutral amines and the molar ratio of fluorophore/monomer, according to first-order reaction kinetics ($v = k * [\text{glucosamine}]_{\text{neutral}} * [\text{RITC}]$). Chitosans with distinct DDA and molecular weight were equivalently labeled using chitosans degraded either with nitrous acid, which is a very effective and rapid depolymerization agent for chitosan, or acid-heat which also generates sterile parent solutions (Allan & Peyron, 1995; Varum, Ottoy, & Smidsrod, 2001). Each library of RITC-chitosan derivatives had the same relative fluorescence and nearly identical structural properties as the parent chitosans, for ultimate use as *in vivo* fluorescent tracers.

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