The kinetics of chitosan depolymerisation at different temperatures

Gordon A. Morris[a,✉], Jonathan Castile[b], Alan Smith[b], Gary G. Adams[a,c] and Stephen E. Harding[a]

[a]National Centre for Macromolecular Hydrodynamics, School of Biosciences, University of Nottingham, Sutton Bonington, LE12 5RD, U.K.

[b]Archimedes Development Limited, Albert Einstein Centre, Nottingham Science and Technology Park, University Boulevard, Nottingham, NG7 2TN, U.K.

[c]Institute of Clinical Research, University of Nottingham, Faculty of Medicine and Health Science, Clifton Boulevard, Nottingham NG7 2RD, UK

✉Corresponding author

Tel: +44 (0) 115 9516149

Fax: +44 (0) 115 9516142

Email: gordon.morris@nottingham.ac.uk

Abstract

The stability (in terms of molar mass) of chitosan potentially plays an important role in its behaviour and functional properties in a wide range of applications and therefore any changes over time must be understood.

The weight-average molar masses and intrinsic viscosities of chitosan solutions at different temperatures (4 °C, 25 °C and 40 °C) have been investigated using size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) and a "rolling ball" viscometer respectively. The weight-average molar mass (M_w) and the intrinsic viscosity ([η]) both decrease with increased storage time, although this phenomenon is more pronounced at elevated temperatures.

Good correlation was found between the changes in molar mass and intrinsic viscosity with time and these parameters were used to determine the depolymerisation constant (*k*) and the activation energy (E_a).

Knowledge of the effect of storage conditions (*e.g.* temperature) is important in the understanding the stability of chitosan solutions, but whether or not chitosan depolymerisation will be detrimental to its intended application will depend on the functional significance of the changes that occur.

Keywords: Chitosan; molar mass; intrinsic viscosity; stability; kinetics; activation energies

Introduction

Due to being in a unique position as the only "natural" polycationic polymer chitosan and its derivatives have received a great deal of attention from, for example, the food, cosmetic and pharmaceutical industries. Important applications include water and waste treatment, antitumor, antibacterial and anticoagulant properties [1]. The interaction of chitosan with mucus is also important in oral and nasal drug delivery [2].

Chitin (poly-N-acetyl-D-glucosamine) extracted from the exoskeleton of crustaceans or from the mycelia of fungi can be fully or partially deacteylated to produce chitosan [1,3]. In chitosan (**Figure 1**) the N-acetyl group is replaced either fully or partially by NH_2 therefore the degree of acetylation can vary from DA = 0 (fully deacteylated) to DA = 1 (fully acetylated *i.e.* chitin). In partially acetylated chitosans acetylated monomers (GlcNAc; A-unit) and deacteylated monomers (GlcN; D-unit) have been shown to be randomly distributed [4,5].

<Figure 1 here>

The stability (shelf-life) of chitosan in terms of molar mass, viscosity and conformation is highly relevant to its commercial uses as these properties can play an important role in the function of chitosan [6]. It is therefore fundamentally important to have the means available with which to measure the effects of and understand the relationships between storage conditions and stability.

In this paper we will look at the stability of chitosan solutions across a range of different temperature conditions: 4 ºC, 25 ºC and 40 ºC.

Materials and Methods

Samples

Chitosans of differing molar mass, designated as low (L), medium (M) and high (H) mass with degree of acetylation (DA) of ~ 20 % were obtained from Novamatrix (Oslo, Norway) and from Sigma Chemical Company (St. Louis, U.S.A.) and were used without any further purification. Chitosans (100 mg) were dissolved in 0.2 M pH 4.3 acetate buffer (100 ml) with stirring for 16 hours. The stability of chitosan solutions was then determined by measuring the weight average molar mass, M_w and intrinsic viscosity, [η] at different times for up to 12 months at 4 ºC, 25 ºC or 40 ºC.

Viscometry

The densities and viscosities of sample solutions and reference solvents were analysed using an AMVn Automated Micro Viscometer and DMA 5000 Density Meter (both Anton Paar, Graz, Austria) under precise temperature control (20.00 ± 0.01 ºC). The relative, η_{rel} and specific viscosities, η_{sp} were calculated as follows:

$$\eta_{rel} = \left(\frac{\eta}{\eta_0} \right) \tag{1}$$

$$\eta_{sp} = \eta_{rel} - 1 \tag{2}$$

Where η is the dynamic viscosity (*i.e.* corrected for density) of a chitosan solution and η_o is the dynamic viscosity of buffer (1.0299 mPas).

Measurements were made at a single concentration (~ 1.0 x 10^{-3} g ml^{-1}) and intrinsic viscosities, [η], were estimated using the Solomon-Ciutâ approximation [7].

$$[\eta] \approx \frac{\left(2\eta_{sp} - 2\ln(\eta_{rel})\right)^{1/2}}{c} \tag{3}$$

Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-MALLS)

Analytical fractionation was carried out using a series of SEC columns TSK G6000PW, TSK G5000PW and TSK G4000PW protected by a similarly packed guard column (Tosoh Bioscience, Tokyo, Japan) with on-line MALLS (Dawn DSP, Wyatt Technology, Santa Barbara, U.S.A.) and refractive index (Optilab rEX, Wyatt Technology, Santa Barbara, U.S.A.) detectors. The eluent (0.2 M pH 4.3 acetate buffer) was pumped at 0.8 ml min^{-1} (PU-1580, Jasco Corporation, Great Dunmow, U.K.) and the injected volume was 100 µl (~1.0 x 10^{-3} g ml^{-1}) for each sample. Absolute weight-average molar masses (M_w) were calculated using the ASTRA® (Version 5.1.9.1) software (Wyatt Technology, Santa Barbara, U.S.A.), using the refractive index increment, dn/dc = 0.163 ml g^{-1} [8].

Sedimentation Velocity in the Analytical Ultracentrifuge

Sedimentation velocity experiments were performed using an Optima XLI Analytical Ultracentrifuge (Beckman Instruments, Palo Alto, U.S.A.). Chitosan solutions (~ 1.0 x 10^{-3} g ml^{-1}) and 0.2 M pH 4.3 acetate buffer were injected into the solution and reference channels, respectively of a double sector 12 mm optical path length cell. Samples were centrifuged at 45000 rpm at a temperature of 20.0 ºC. Concentration profiles and the movement of the sedimenting boundary in the analytical ultracentrifuge cell were recorded using the Rayleigh interference optical system and converted to concentration (in units of fringe displacement relative to the meniscus, j) versus radial position, r [9]. The data was then analysed using the "least squares, ls-g(s) model" incorporated into the SEDFIT (Version 9.4b) program [10,11]. This software generates an apparent distribution of sedimentation coefficients in the form of ls-$g^*(s)$ versus s^*, where the * indicates that the distribution of sedimentation coefficients have not been corrected for diffusion effects [9].

Results and Discussion

Intrinsic viscosity and molar mass

Intrinsic viscosities and weight-average molar masses (**Tables 1 - 3**) of chitosans (L, M and H) generally decrease with increased storage time. The effect becomes more marked as storage temperature is increased.

<Tables 1 - 3 here>

Sedimentation coefficient

The sedimentation coefficient was measured prior to storage at different temperatures and after 12 months at 4 ºC, 25 ºC and 40 ºC. As we can see from **Tables 1 – 3** the sedimentation coefficient is essentially insensitive to molar mass. This is typical of semi-flexible coil and rod type molecules, where the Mark-Houwink-Kuhn-Sakurada (MHKS) power law exponent, *b*, can be as low as 0.15 [3,9] and therefore a 2-fold increase in molar mass may result in only a 1.1-fold ($2^{0.15}$) increase in sedimentation coefficient. We should also note that the sedimentation coefficients have not been corrected for their concentration dependencies [12], k_s, which can make a great difference for rigid polymers of high molar mass [3,9]. This may therefore result in an *apparent* increase in sedimentation coefficient at finite concentration, although the molar mass and sedimentation coefficient at infinite dilution have decreased. We can however see from **Figure 2** that although the sedimentation coefficient remains unchanged the width of the peak changes greatly. The width of the peak depends on both diffusion and polydispersity; however for rigid polymers the influence of polydispersity is minimal as the sedimentation coefficient is insensitive to changes in molar mass [3,9]. Taking this into account we confirm the decrease in molar mass by an increase in diffusion (if the sedimentation coefficient remains constant) via the Svedberg equation [13].

$$M_w = \frac{s^0 RT}{D^0(1-\bar{v}\rho)} \tag{4}$$

Where s^0 and D^0 are the sedimentation and diffusion coefficients respectively at infinite dilution; R is the universal gas constant (8.314 x 10^7 erg K^{-1} mol^{-1}); T is the

absolute temperature (293 K); ρ is density of the solvent (1.0083 g ml^{-1}) and $\bar{v}$ (= 0.57 ml g^{-1}) is the partial specific volume of chitosan [14].

<Figure 2 here>

Kinetics and activation energy of chitosan depolymerisation

If depolymerisation follows 1st order kinetics the degradation rate constant (*k*) can be calculated from the following equation [15].

$$\left(\frac{1}{M_{w,t}}\right)-\left(\frac{1}{M_{w,t=0}}\right)=\left(\frac{k}{m}\right)t \quad (5)$$

Where $M_{w,t=0}$ and $M_{w,t}$ are the weight-average molar masses, *t* is time in days and *m* is the molar mass an average chitosan monomer = 216 g mol^{-1} [16,17].

We can also convert intrinsic viscosities in to molar mass using the following Mark-Houwink-Kuhn-Sakurada (MHKS) power law relationship [18].

$$[\eta]=0.0074\,M_w^{\,0.95} \quad (6)$$

This enables the estimation of the 1st order rate constant (*k*) from both molar mass and intrinsic viscosity measurements (**Figure 3** and **Table 4**).

<Figure 3 and Table 4 here>

We can see that for all three chitosans (**Table 4**) the rate constant is smallest at 4 °C and greatest at 40 °C, although this is less marked for the high molar chitosan (H). The agreement between estimates for ln k from molar mass and intrinsic viscosity is especially good at 40 °C and is quite poor at 4 °C. The rate constants are lower than those found by Holme, Davidsen, Kristiansen, & Smidsrød (2008) [19] at pH 5 which is consistent with decreased depolymerisation at lower pH [20]. The starting molar

mass ($M_{w,t=0}$) does *not* appear to have any influence on the rate of depolymerisation over the range studied.

The first order rate constant (k) can then be used to determine the activation energy (E_a) of depolymerisation using the Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT} \quad (7)$$

Where R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and T is the temperature in Kelvin.

We find, albeit with only 3 data points, an activation energy of 15 – 80 kJ mol^{-1} (**Figure 4** and **Table 5**) which appears to negatively correlated with molar mass and is generally less than the value of ~ 80 kJ mol^{-1} found by Holme et al (2008) [19]. This may be due to lower pH in this study (pH 4.3 vs. pH 5.0) and the possible presence of oxygen in the headspace of the sample container which will promote oxidative-reductive depolymerisation (ORD) in addition to acid depolymerisation [19,20]. Further studies will include an investigation into any chemical changes upon storage *e.g.* formation/ loss of functional groups and the effect of pH and temperature on the physico-chemical properties of chitosan.

<Figure 4 and Table 5 here>

Conclusions

It has been clearly demonstrated that over a range of molar masses chitosan is susceptible to depolymerisation in dilute solution at pH 4.3. This is especially true at higher temperatures (40 °C). Rate constants (at 25 °C and 40 °C) for the depolymerisation of chitosan estimated from changes in either molar mass or intrinsic viscosity show good correlation.

Chitosan storage conditions and particularly temperature may be important but whether or not chitosan depolymerisation will be detrimental to its intended

application will depend on the functional significance of the changes that occur. For example it has been reported that low molar mass chitosans can cause more cell damage [21], although they may also prevent diabetes mellitus progression in mice to a greater extent than high molar chitosans [22], show greater antibacterial activity compared with high molar mass chitosans [23] and whilst the high viscosities of high molar mass chitosans limit its biological usefulness, low molar mass chitosan is more soluble at neutral pH and therefore potentially more available *in vivo* [24]. However it has also been reported that high molar mass chitosans show greater antibacterial activity compared with low molar mass chitosans [25], that nasal insulin delivery [26] is more effective with chitosan of molar mass greater than 100000 g mol^{-1} and the reversibility of transepithelial chemical resistance (TEER) values decrease with decreased chitosan molar mass [27].

The measurement of molar mass and intrinsic viscosity and estimation of rate constant and activation energy provide a ready means to quantify chitosan depolymerisation, and may be used to determine the role of other factors such as pH, light and chemical modification on stability, although this alone is not sufficient to make any conclusions about their performance in a particular application.

Acknowledgements

We thank the United Kingdom Biotechnology and Biological Sciences Research Council (BBSRC) for their financial support.

References

1. Rinaudo M. Chitin and chitosan: properties and applications. Prog Polym Sci 2006; 31:603-632.
2. Harding SE, Davis SS Deacon MP, Fiebrig I. Biopolymer mucoadhensives. In: Harding SE, editor. Biotechnology and Genetic Engineering Reviews Vol. 16. Andover: Intercept, 1999. p. 41.
3. Tombs MP, Harding SE, Polysaccharide biotechnology. London: Taylor Francis, 1998.
4. Vårum KM, Anthonsen MW, Grasdalen H, Smidsrød O. Determination of the degree of N-acetylation and distribution of N-acetyl groups in partially N-deacteylated chitins (chitosans) by high-field NMR spectroscopy. Carbohyd Res 1991; 211:17-23.
5. Vårum KM, Anthonsen MW, Grasdalen H, Smidsrød O. ^{13}C NMR studies of the acetylation sequences in partially N-deacteylated chitins (chitosans), Carbohyd Res 1991; 217:19-27.
6. Skaugrud O, Hagen A, Borgersen B, Dornish M. Biomedical and pharmaceutical applications of alginate and chitosan. In: Harding SE, editor. Biotechnology and Genetic Engineering Reviews Vol. 16. Andover: Intercept, 1999. p.23.
7. Solomon OF, Ciutâ IZ. Détermination de la viscosité intrinsèque de solutions de polymères par une simple détermination de la viscosité. J Appl Polym Sci 1962; 24:683-686.
8. Rinaudo M. Milas M, Le Dung P. Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. Int J Biol Macromol 1993; 15:281-285.
9. Harding SE. Analysis of polysaccharide size, shape and interactions. In: Scott DJ, Harding SE, Rowe AJ, editors, Analytical ultracentrifugation techniques and methods. Cambridge: Royal Society of Chemistry, 2005. p. 231.
10. Schuck P. Sedimentation analysis of non-interacting and self-associating solutes using numerical solutions to the Lamm equation. Biophys J 1998; 75:1503-1512.

11. Schuck P. Diffusion-deconvoluted sedimentation coefficient distributions for the analysis of interacting and non-interacting protein mixtures. In: Scott DJ, Harding SE, Rowe AJ, editors, Analytical ultracentrifugation techniques and methods. Cambridge: Royal Society of Chemistry, 2005. p. 26.
12. Gralén N. Sedimentation and diffusion measurements on cellulose and cellulose derivatives, PhD Dissertation, University of Uppsala, Sweden, 1944.
13. Svedberg T, Pedersen KO. The ultracentrifuge. Oxford: Oxford University Press, 1940.
14. Errington N, Harding SE, Vårum KM Illum L. Hydrodynamic characterisation of chitosans varying in degree of acetylation. Int J Biol Macromol 1993**:** 15**:**113-117.
15. Zhou G, Yao W. Wang C. Kinetics of microwave degradation of λ-carrageenan from *Chondrus ocellatus.* Carbohyd Polym 2006; 64:73-77.
16. Vold IMN. Periodate Oxidised Chitosans: Structure and solution properties, PhD Dissertation, Norwegian University of Science and Technology, Trondheim, Norway, 2004.
17. Yamada S, Ganno T, Ohara N, Hayashi Y. Chitosan monomer accelerates alkaline phosphatase activity on human osteoblastic cells under hypofunctional conditions. J Biomed Mater Res A 2007; 83A:290-295.
18. Morris GA, Castile J, Smith A, Adams GG, Harding SE. Macromolecular conformation of chitosan in dilute solution: a new global hydrodynamic approach. Carbohyd Polym 2009; 76:616-621.
19. Holme HK, Davidsen L, Kristiansen A, Smidsrød O. Kinetics and mechanisms of the depolymerization of alginate and chitosan in aqueous solution. Carbohyd Polym 2008; 73:656-664.
20. M. Fee, Evaluation of chitosan stability in aqueous systems, *PhD Dissertation*, University of Nottingham, United Kingdom,2005.
21. Aspden TJ, Illum L, Skaugrud O. Chitosan as a nasal delivery system: evaluation of insulin absorption enhancement and effect on nasal membrane integrity using rat models. Eur J Pharm Sci 1996; 4:23-31.
22. Kondo Y, Nakatani A, Hayashi K Ito M. Low molecular weight chitosan prevents the progression of low dose streptozotocin induced slowly progressive diabetes mellitus in mice. Biol Pharm Bull 2000; 23:1458-1464.

23. Liu XF, Guan YL, Yang DZ, Li Z, De Yao K. Antibacterial action of chitosan and carboxymethylated chitosan. J Appl Polym Sci 2001; 79:1324-1335.
24. Harish Prashanth KV, Tharanathan RN. Chitin/ chitosan: modifications and their unlimited potential – an overview. Trends Food Sci Tech 2007; 18:117-131.
25. No HK, Kim SH, Lee SH, Park NY, Prinyawiwatkul W. Stability and antibacterial activity of chitosan solutions affected by storage temperature and time. Carbohyd Polym 2006; 65:174-178.
26. Davis SS, Illum L. Chitosan for oral drug delivery, In: Muzzarelli RAA, editor. Chitosan per os: from dietary supplement to drug carrier. Grottammare: Atec, 2000. p. 137.
27. Holme HK, Hagen A, Dornish M. Influence of chitosans with various molecular weights and degrees on deacetylation on the permeability of human intestinal epithelial cells (Caco-2), In: Muzzarelli RAA, editor. Chitosan per os: from dietary supplement to drug carrier. Grottammare: Atec, 2000. p. 127.

Table 1 Solution properties for a low molar mass chitosan (L) in 0.2 M pH 4.3 acetate buffer

Storage Time (days)	**Storage Temperature (°C)**	**[η] (ml g^{-1})**	**M_w (g mol^{-1})**	**s* (S)**
0	-	465 ± 15	115000 ± 5000	1.19 ± 0.05
31	4	490 ± 15	115000 ± 5000	
60	4	460 ± 15	115000 ± 5000	
158	4	440 ± 15	130000 ± 5000	
355	4	430 ± 5	110000 ± 5000	1.37 ± 0.05
31	25	430 ± 15	105000 ± 5000	
60	25	415 ± 10	100000 ± 5000	
158	25	355 ± 10	105000 ± 5000	
355	25	300 ± 5	90000 ± 5000	1.22 ± 0.05
31	40	345 ± 10	75000 ± 5000	
60	40	320 ± 10	70000 ± 5000	
158	40	270 ± 10	65000 ± 5000	
355	40	215 ± 5	45000 ± 5000	1.22 ± 0.05

Table 2 Solution properties for a medium molar mass chitosan (M) in 0.2 M pH 4.3 acetate buffer

Storage Time (days)	**Storage Temperature (°C)**	**[η] (ml g^{-1})**	**M_w (g mol^{-1})**	**s* (S)**
0	-	1450 ± 40	290000 ± 20000	1.15 ± 0.05
31	4	1185 ± 35	260000 ± 20000	
60	4	1125 ± 35	270000 ± 20000	
158	4	1020 ± 30	270000 ± 20000	
355	4	910 ± 25	130000 ± 10000	1.37 ± 0.06
31	25	1180 ± 35	290000 ± 15000	
60	25	1075 ± 30	290000 ± 20000	
158	25	925 ± 30	235000 ± 20000	
355	25	735 ± 20	115000 ± 10000	1.37 ± 0.06
31	40	960 ± 30	225000 ± 20000	
60	40	815 ± 25	195000 ± 20000	
158	40	655 ± 20	160000 ± 5000	
355	40	515 ± 15	100000 ± 5000	1.35 ± 0.05

Table 3 Solution properties for a high molar mass chitosan (H) in 0.2 M pH 4.3 acetate buffer

Storage Time (days)	**Storage Temperature (°C)**	**[η] (ml g^{-1})**	**M_w (g mol^{-1})**	**s* (S)**
0	-	1765 ± 55	425000 ± 20000	1.10 ± 0.05
31	4	1530 ± 45	380000 ± 20000	
60	4	1350 ± 40	400000 ± 15000	
158	4	1175 ± 35	340000 ± 5000	
355	4	1015 ± 15	150000 ± 5000	1.10 ± 0.05
31	25	1210 ± 35	320000 ± 15000	
60	25	1120 ± 35	320000 ± 10000	
158	25	825 ± 25	230000 ± 5000	
355	25	665 ± 10	105000 ± 5000	1.07 ± 0.05
31	40	845 ± 25	205000 ± 5000	
60	40	745 ± 20	175000 ± 5000	
158	40	555 ± 15	130000 ± 5000	
355	40	405 ± 5	80000 ± 5000	1.01 ± 0.05

Table 4 Kinetic rate constants* (day^{-1}) for low, medium and high molar mass chitosans at 4 ºC, 25 ºC and 40 ºC from molar mass and intrinsic viscosity determinations

Chitosan	Storage Temperature (ºC)					
	4		25		40	
	ln k (from M_w)	ln k (from [η])	ln k (from M_w)	ln k (from [η])	ln k (from M_w)	ln k (from [η])
L	-15.8	-14.0	-13.7	-12.4	-11.9	-11.8
M	-15.6	-13.7	-12.6	-13.1	-12.5	-12.5
H	-12.9	-13.6	-12.4	-12.8	-12.1	-12.1

*presented in the form of the natural log (ln)

Table 5 Activation energies for the depolymerisation of chitosan from molar mass and intrinsic viscosity determinations

Chitosan	E_a (kJ mol^{-1})	
	M_w	[η]
L	77 ± 7	45 ± 6
M	65 ± 25	24 ± 3
H	16 ± 1	30 ± 3

Legends to Figures

Figure 1 schematic representation of the structure repeat units of chitosan, where R = Ac (GlcNAc; A-unit) or H (GlcN; D-unit) depending on the degree of acetylation.

Figure 2 sedimentation coefficient distributions for a high molar mass chitosan (H) at time 0 (−) and after 12 months at 4 ºC (−), 25 ºC (−) and 40 ºC (−).

Figure 3 1st order kinetic plots of (mol g^{-1}) vs. time (days) for:

A – low molar mass chitosan (L)

B – medium molar mass chitosan (M)

C – high molar mass chitosan (H)

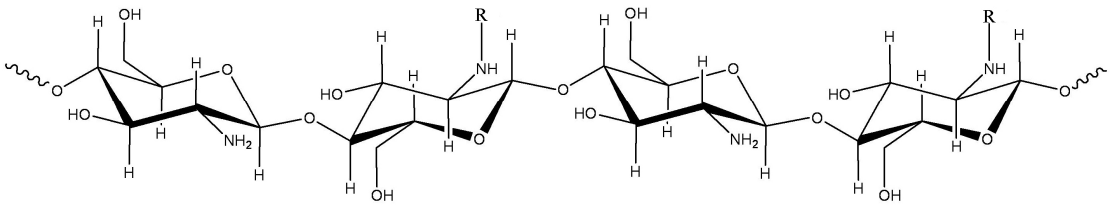
where open and closed symbols represent molar masses estimated from viscometry and SEC-MALLS respectively at 4 °C (∀, !), 25 °C (–, ,) and 40 °C (8, 7).

Figure 4 Arrhenius plots for the depolymerisation of chitosan. Where the 2 extrapolated lines represent the natural log (ln) of the initial rate constants (*k*) calculated from SEC-MALLS (black) and intrinsic viscosity (red) versus reciprocal temperature (1/T). The activation energy (E_a) is calculated from the slope (equation 5):

A – low molar mass chitosan (L)

B – medium molar mass chitosan (M)

C – high molar mass chitosan (H)



D A (if R = Ac) D A (if R = Ac)

Figure 1

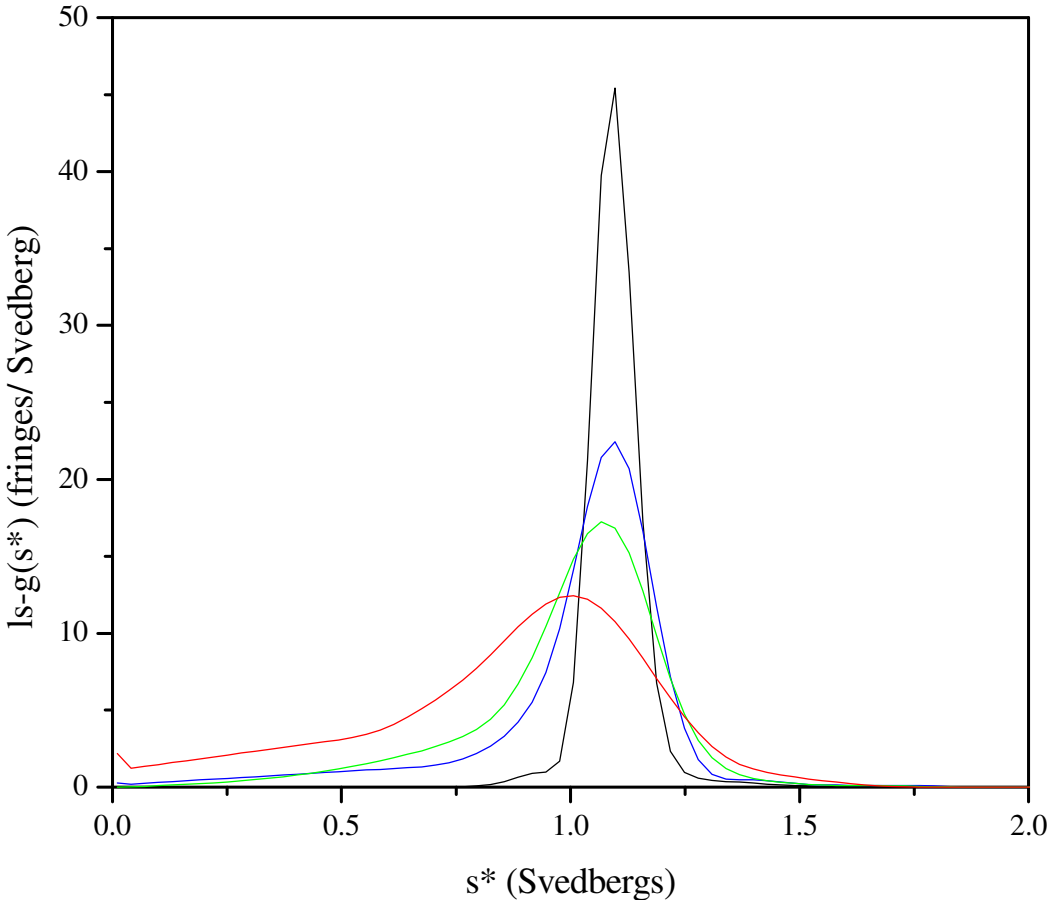


Figure 2

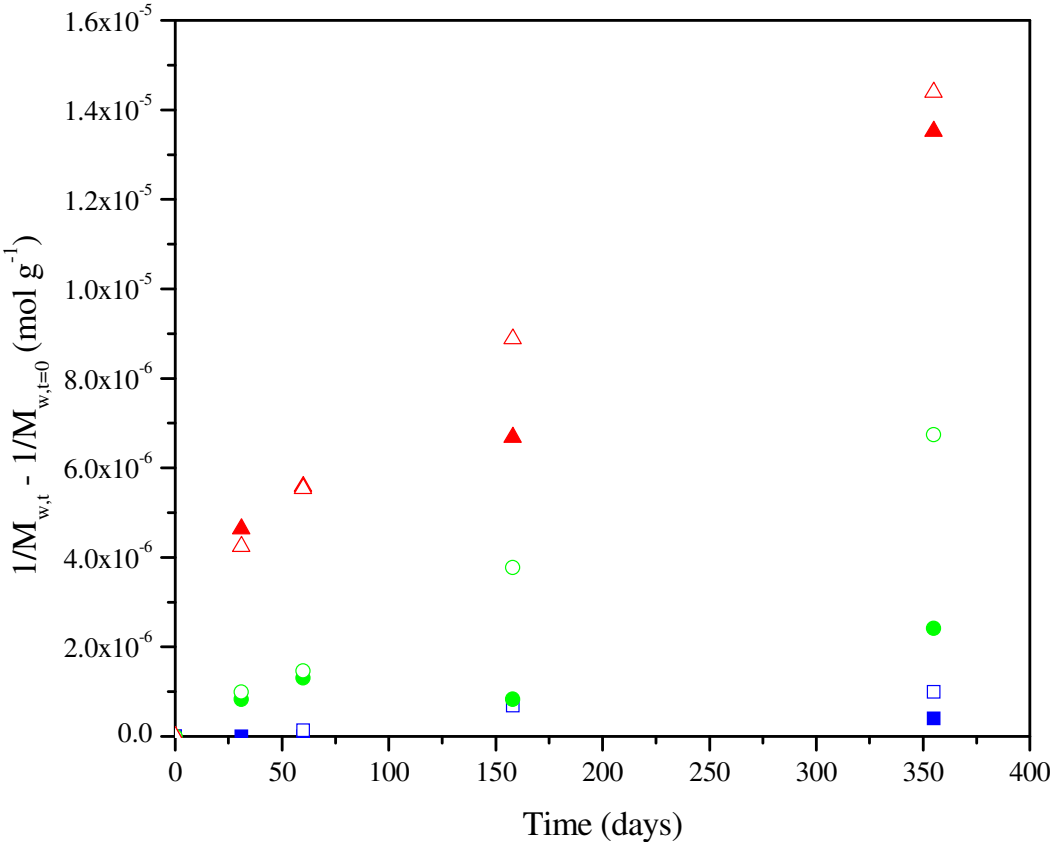


Figure 3A

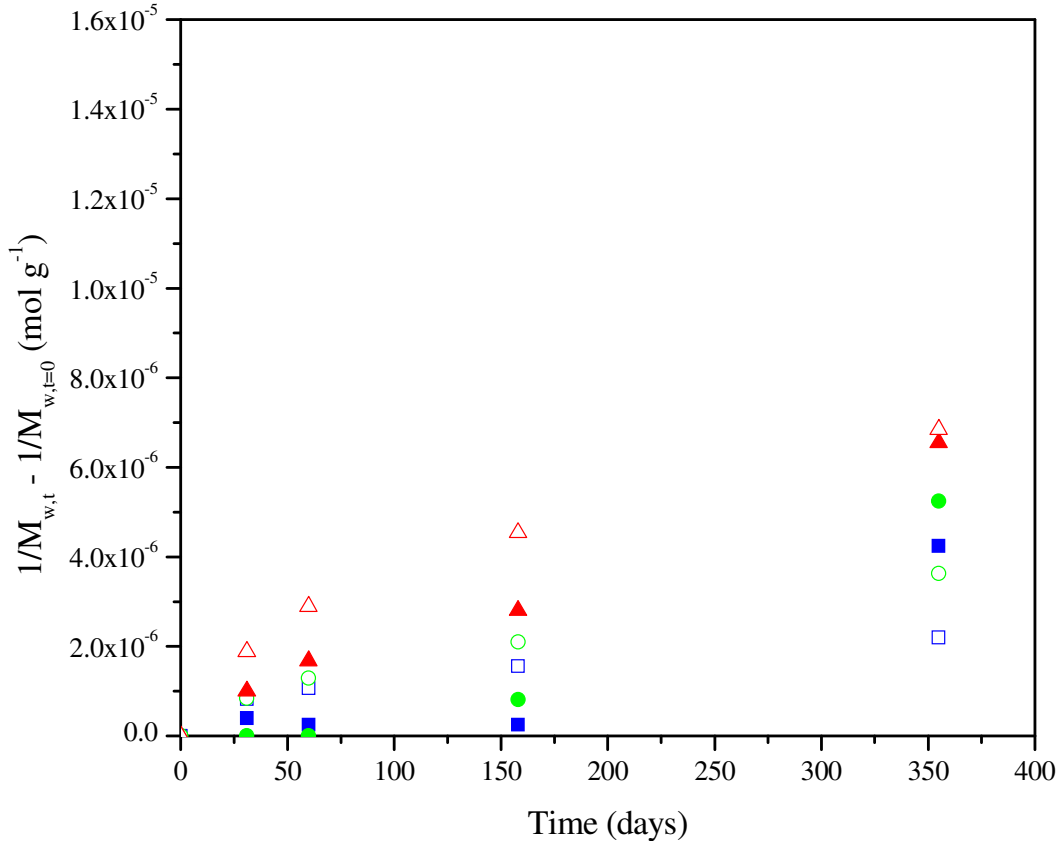


Figure 3B

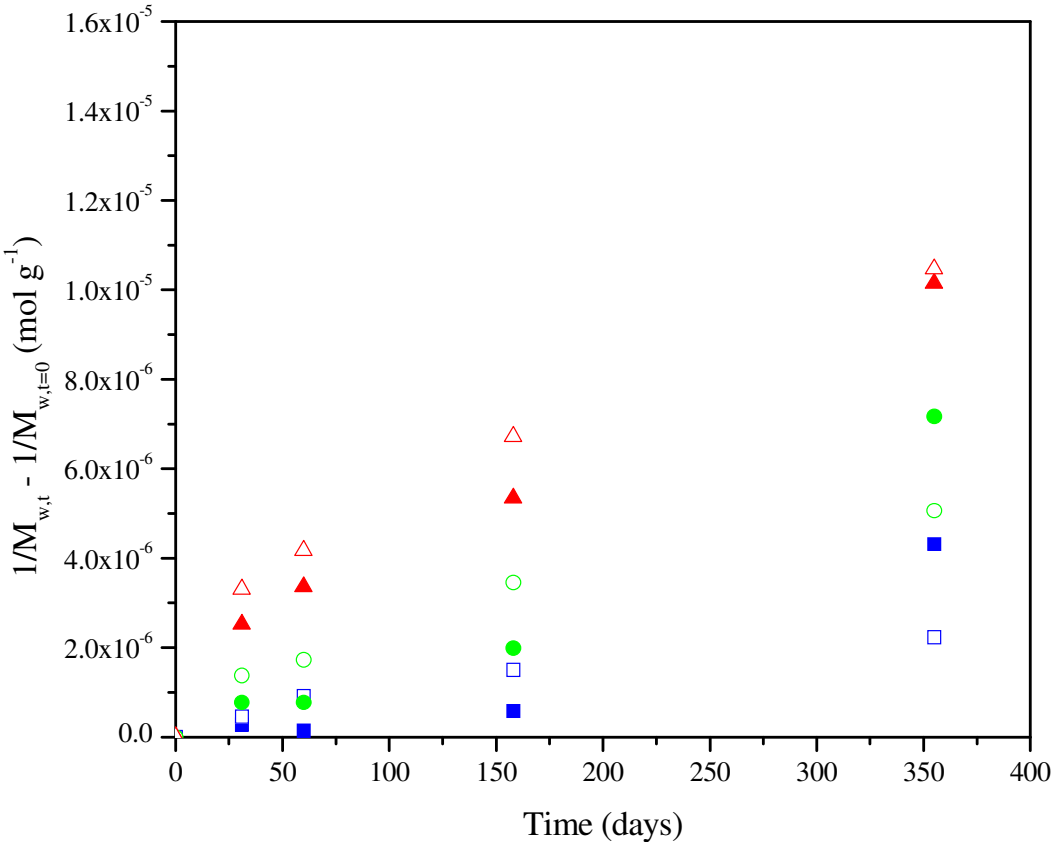

1.6x10-5
1.4x10-5
1.2x10-5
1.0x10-5
8.0x10-6
6.0x10-6
4.0x10-6
2.0x10-6
0.0
1/Mw,t - 1/Mw,t=0 (mol g-1)
0
50
100
150
200
250
300
350
400
Time (days)

Figure 3C

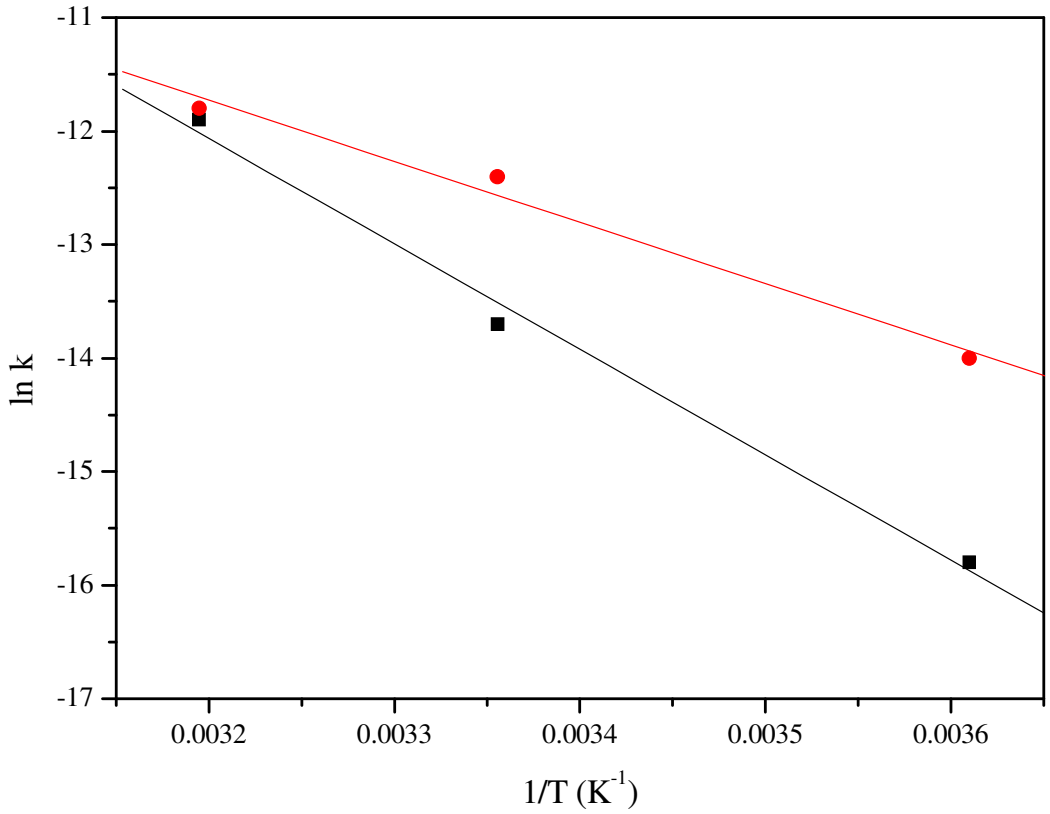


Figure 4A

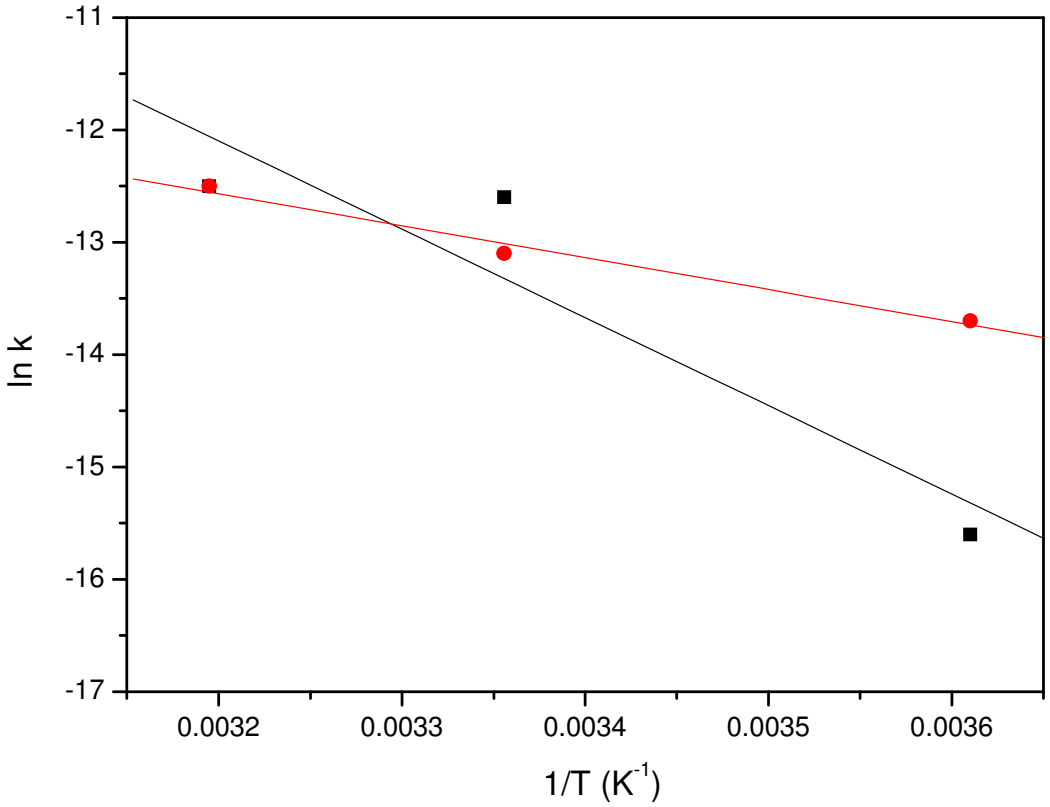


Figure 4B

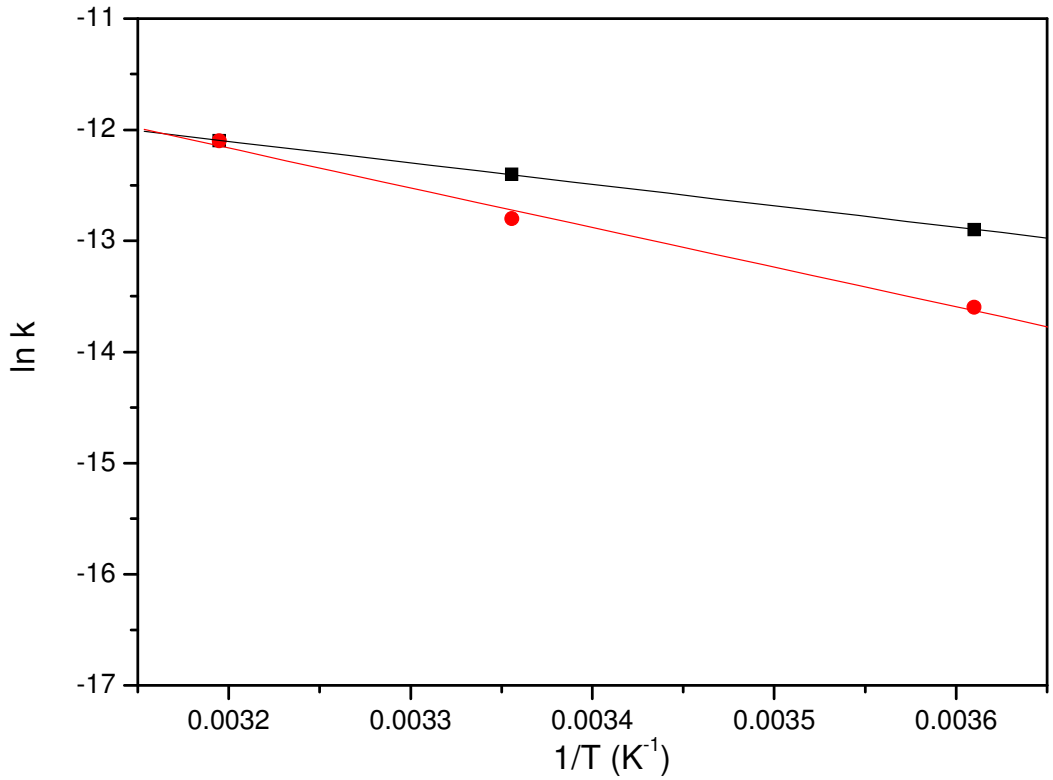


Figure 4C