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Structure-property relationships in chitosan and selected film-forming derivatives

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This thesis is submitted in partial fulfilment of
the requirements for the award of a PhD

March 2002

Abstract

Converting chitosan into a recently patented polyanion in a confirmed non-degradative process constituted an improved method for determining molecular weight of chitosan by SEC-MALLS, previous problems with the polycation adsorbing onto columns with resulting inaccurate molecular weights being eliminated. From these reliable molecular weights the degree of polymerisation for the novel polymer and hence for chitosan were calculated and improved Mark-Houwink relationships for both polymers in a range of fraction of *N*-acetylation (F_A) of 0.148 to 0.609 were established. The limiting viscosity number values, α parameter relationships and Huggins constants, when regarded as functions of F_A, are indices for conformational changes of the copolymer continua. For chitosan three stages were found, from an electrostatically controlled extended coil at constant and fairly high flexibility in the F_A range of approximately 0.0 to 0.25 to an progressively stiffening more rod-shaped conformation up to about 0.5, beyond which the stiffness again decreases together with the hydrodynamic volume. For the anionic derivative an overall inverse relationship of hydrodynamic volume and F_A could be established. Its shape bears rod-like characteristics at low F_A changing to increasingly random coil.

Different routes of preparing water/pH resistant chitosan-based membranes involved either hydrophobic substitution to increase organo-solubility in simple solvents like acetone or crosslinking with the environment- and bio-friendly crosslinking reagent citric acid. Promising results were obtained from both methods in the form of a novel organo-soluble chitosan derivative and a crosslinked product of chitosan with favourable acid resistance.

The scope of dye interaction for absolute compositional analysis was extended to complex systems of linear substitution as well as estimation of degree of crosslinking. The efficiency of *N*-acetylation was improved and a method for predicting anhydride amounts required for producing defined reacetylated material was established. The stability of chitin gels was influenced by F_A, molecular weight and co-solvent proportions.The so far unpublished structurally strongly chitin-related *N*-carbamylchitosan exhibited similar solubility but different gelation behaviour.

to my big brother Robert

to Ernest Shackleton for his inspiring example of leadership
to Lise Meitner for her exemplary integrity

Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself (1).

If you want to understand function, study structure. (2).

(Francis Harry Compton Crick,
English Biophysicist, 1988,
What Mad Pursuit)

Acknowledgements

I would like to express my sincerest and most heart felt gratitude to all those who contributed to the completion of my PhD research project and thesis.

- The Nottingham Trent University Research Office for funding
- Membrane Systems Ltd., UK for a partial grant and contribution of funding for essential equipment
- NTTS for use of additional equipment and facilities
- Independent Fisheries, New Zealand for providing squid pen

- Prof. George A. F. Roberts for long term mentoring during my two undergraduate research projects and during my PhD, his excellent supervision, his inspiring faith in my work and his introducing me to the wonders of complex carbohydrates and especially chitin and chitosan
- Fran Wood for her can-do attitude and her resourcefulness, as well as her friendship
- Colleagues Angela Bartkowiak, Dr. Wei Wang, Dr. Chiao-Chung (Tina) Huang, Judith Kerrigan and last but not least Simon Mazengarb for being good colleagues and friends
- Dr. John Hearn for his fresh-minded input, support and supervision
- Prof. Alain Domard (Director, Laboratoire des Materiaux Polymeres et des Biomateriaux, Université Claude Bernard, France) for unceasing support collaboratively pioneering the characterisation of a novel water soluble chitosan derivative by SEC-MALLS and his technician Jean-Michel Lucas for carrying out the analysis
- Gary Ostroff, PhD (VP R&D, Biopolymer Engineering Inc., USA) for stretching my mind and challenging discussions as well as making time for having many crucial chitosan samples analysed by SEC-MALLS
- Malcolm Coffey (TNTU Chemistry &Physics) for sharing resources with our group

- Relatives and friends in Germany, Britain and other places for sanity breaks and counsel
- My friend/housemate/landlady Clare for putting up with me and for her support and friendship
- My late grandmother Barbara for encouraging my growth, my parents Karl and Marga for supporting my educational choices
- My big brother Robert for his encouragement, support and for walking ahead of me

Glossary of abbreviations

Abbreviation	Explanation	Comment
Ac_2O	Acetic anhydride	
AO7	Acid Orange 7	
BB9	C.I. Basic Blue 9, Methylene Blue	
Bu_2O	Butyric anhydride	
CH	Chitosan	
Cha, b, c	Chitosans from different sources	
CHMS	Sodium *N*-methylsulphonated chitosan	
CHMS-X [0.YZ]	Sodium *N*-methylsulphonated chitosan from chitosan of MW fraction X and degree of acetylation [0.YZ]	E.g. CHMS-H[0.25] is CHMS produced by conversion of chitosan of MW fraction high (H) with a fraction of *N*-acetylation of 0.25
C.I.	Colour index	Published by the Society of Dyers and Colourists
D_A	Degree of *N*-acetylation	/[%]
DP	Average degree of polymerisation	
DPD	Distribution of degree of polymerisation	
DMAc	Dimethylacetamide	
DTPA	3,3'-Dithiodipropionic acid	
EP	Equivalence point (metachromatic titration)	
EtOH	Ethanol	
EW	Equivalent weight	/[g/mol]
F_A	Fraction of *N*-acetylation	
F_C	Fraction of *N*-carbamylation	
F_H	Fraction of *N*-hexanoylation	
F_S	Fraction of *N*-substitution	
FSBS	Formaldehyde sodium bisulfite addition compound, CH_3NaO_4S	
Hex_2O	Hexanoic anhydride	
HMW	High molecular weight	
HOAc	Acetic acid	
K_H	Huggins constant	
LVN	Limiting viscosity number	/[mL/g]
m_0	Average molecular weight of one sugar unit	
MeOH	Methanol	
M_v	Viscosity average molecular weight	
MW	Average molecular weight	
MWD	Molecular weight distribution	
NaOAc	Sodium acetate	
PD	Polydispersity	
RT	Room (ambient) temperature	18°C - 28°C
SDC	Society of Dyers and Colourists	

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1. Introduction

1.1 General

1.1.1 Potential and historical development

After cellulose, chitin is the living world's most important polysaccharide as well as being the second most abundant biomaterial produced per year. Mostly found in the exoskeleton of crustaceans and insects as well as in the cell walls of fungi, an estimated figure of the world-wide annual bioproduction is more than 10^9 t (8) with a stable catch volume of crustaceans of $2.5*10^6$ t in recent years (9).

With chitin being extracted from fishery waste with a high yearly chitin potential, it seems obvious why this polymer is attracting so much interest in finding new fields of use. But one should not be deceived, although it is produced from readily available waste in high amounts, chitin and chitosan are comparatively expensive materials because of their costly preparation processes. The polymers are required for different purposes and thus produced in a wide range of different qualities. Reproducibly manufactured and well-characterised chitin and chitosan are only indicated for "high-value-in-use utilisation" (8) like e.g. food supplements and medical applications, while inexpensive chitin and chitosan is useful in e.g. adsorption and flocculation processes for effluent treatment for purification and material recovery (10).

Chitin is the source for its soluble and more reactive derivative chitosan, produced by de-*N*-acetylation of the former. Considering that in 1986 in Japan only 3.2 % of the annual consumption of 1,270 t was actually used as chitin itself, chitosan appears to be the more important reagent or material. Of the chitin produced 92 % was converted to chitosan and a further 4.8 % were used for producing glucosamine and oligosaccharides (11).

Various chitin and chitosan scientists have comprehensively reviewed the historical discovery and development of chitin and chitosan (8, 12-14) so only a brief summary of the time coherence shall be given in the following.

Braconnot, who in 1811 called it “fungine” with it being the alkali resistant residue of some higher fungi, made the initial discovery of chitin. He recognised its different nature from the woody fibres of plants, namely cellulose, which would still take 30 years to be discovered after “fungine”- chitin.

Extracting the exoskeleton of the May beetle, Odier obtained a similar insoluble residue in 1823. He did compare the two materials but assuming that woody material is the only

possible structural component for plants failed to link his newly found substance with Braconnot's fungine since it belonged in the plant kingdom. Finding it in crustacean shells he suggested it to be the basic material of the arthropodal exoskeleton and gave it the actual name chitin from the Greek word for tunic or covering.

Finally in 1859 Rouget reported a product, which he called modified chitin. The material was prepared by treating chitin with KOH under reflux, and described as soluble in dilute organic acids. "Modified chitin" got its modern name from Hoppe-Seyler in 1894, who heated chitin from arthropodal exoskeletons with KOH to 180 °C and thereby produced chitosan. He reported it as soluble in organic and inorganic acids but did not refer to Rouget's earlier work.

With chitosan being so closely related to chitin as its de-*N*-acetylated derivative and cellulose only just being discovered, the three polymers caused considerable confusion and misunderstanding, particularly as the structures of all three were not yet known. It would still take some 40 years into the 20th century to associate chitin and chitosan from plants and animals as the same principal polymer only varying in their parameters such as degree of *N*-acetylation, chain length distribution and their matrices.

A first review on the occurrence and distribution in species was given by Van Wisselingh in 1898 followed by numerous others (12).

Even though chitosan has been known for well over a hundred years it is only during the last 20 years that it has been produced commercially with shellfish waste getting more available.

For some 50 years research on chitin was mostly concerned with occurrence in living organisms, its enzymatic degradation and few applications. Looking at a bibliography (13) one can easily see that from 1950 the number of publications on chitin and chitosan seems to proliferate and the increase of people concerned with these polymers seems to accelerate. In 1977 the first *International Conference on Chitin and Chitosan* was held as the first interdisciplinary meeting of members of the "chitin-world". In order to achieve a greater overall understanding of this versatile natural polymer and to release it from its unjustifiable secondary role in chemistry it seemed to be necessary to assemble the scattered information collected by scientists and industrialists from all over the world. At present the *International Conference on Chitin and Chitosan* takes place in a cycle of three years. Further regular international meetings and societies such as the *International Conference of the European Chitin Society (EUCHIS)*, the *Asia-Pacific Chitin and*

Chitosan Symposium, the *Japanese Chitin Society* and even more recently the *Latin American Society on Chitin and Chitosan (ISOLAQ)* to name just some of the most important examples, have been brought into being.

1.1.2 Structure and nomenclature of chitin and chitosan

Chitosan is a basic polysaccharide soluble in dilute acids with an intermediate composition between [β-(1→4)-2-amino-2-desoxy-D-glucopyranose] (idealised chitosan – Figure 1.1) and [β-(1→4)-2-acetamido-2-desoxy-D-glucopyranose] (idealised chitin – Figure 1.2). Chitosan is typically derived from chitin by alkaline de-*N*-acetylation. (12, 15) but enzymatic methods (16-18) and more recently electrochemical processing have been reported (19-21). Chitin is the structural component of the exoskeletons of crustaceans like shrimp, lobster and others, as well as some fungi (22) where it is also present in the de-*N*-acetylated chitosan form. Crustacean shell is readily available from fishery waste in high amounts (8). Classic methods of extracting chitin from its mostly protein and mineral matrix involve treatment with alkali and mineral acid to remove protein as well as calcium salts (12) and enzymatic methods have been developed (23-25). The advantages of the latter are that the decalcification is mainly achieved through *in situ* production of lactic acid as a consequence of the enzymatic deproteination activity. This results in more valuable by-products such as protein and pigments as opposed to alkaline effluent and is thus an environmentally less harmful process with lower energy requirements. Additionally crustacean waste preservation is accomplished due to lactic acid production (23).

Both are structurally very similar to cellulose and all three only differ in the C(2) position, where cellulose has got a hydroxyl group compared to chitosan's amine and chitin's acetamido group.

The amine group is what makes chitosan especially interesting for chemical modification. While chitin's acetamido group is very stable and cellulose only possesses another hydroxyl group as reactive site, in chitosan different substituents can be introduced on its amine group and on its hydroxyl sites regioselectively.

Chitin has been proved by X-ray diffraction to possess a highly crystalline structure, which is also reflected in its resistance to solubilisation, chemical reaction and enzymatic degradation. Polymorphic forms α-, β- chitin have been confirmed. These forms differ in their orientation of chains, and thus in their stability. β-Chitin's chains are parallel while α-

Figure 1.1: Idealised structure of chitosan

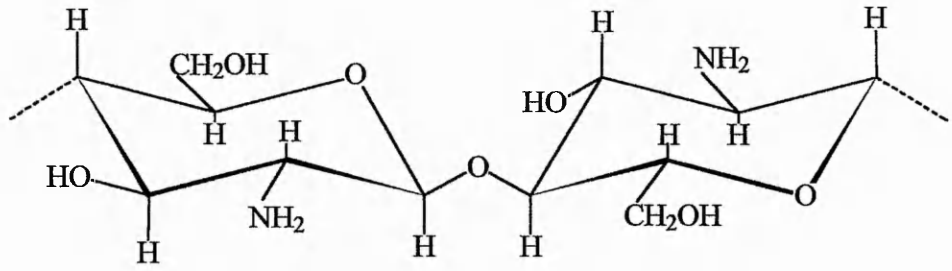
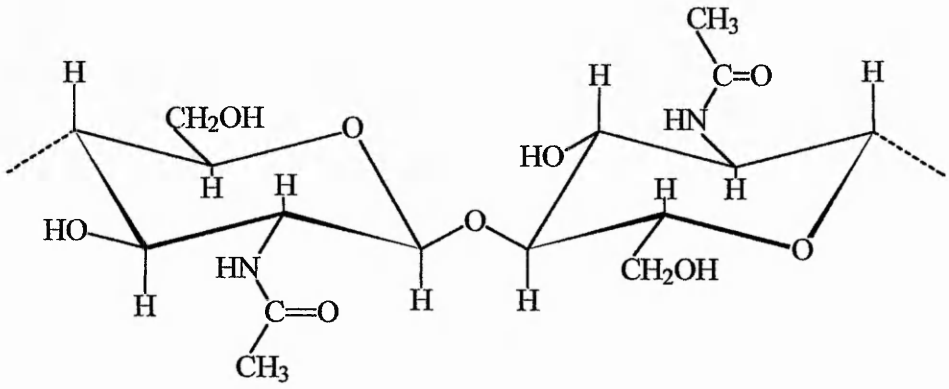


Figure 1.2: Idealised structure of chitin



chitin's chains are oriented antiparallel. The former can be converted to α-chitin, which is the more stable and abundant form (26). α-Chitin is harder than β-chitin, which is more flexible and tougher. In nature the different forms are found in a diversity of functions, sometimes coexisting in the same species.

Chitosan as the de-*N*-acetylated form of chitin does not exist in a crystalline form in nature. Crystalline chitosan is laboratory produced, thus it is not possible to name a single defined structure. The structure-generating material employed for crystallinity assessment can be varied, as a consequence of different polymeric starting materials, different methods of isolation and preparation, and different methods of preparing the chitosan crystals. Furthermore the amount of water of crystallinity present and the possibility of chitosan being uncharged or charged compensated with a variety of possible counterions have an important influence on the resulting crystal structure (27). Thus a number of different

structures have been found. The findings of crystalline structures for chitin and chitosan have been comprehensively reviewed (12). Again crystallinity is accountable for the lack of solubility of chitosan in water.

It has been elucidated that the terms chitin and chitosan are used for copolymers with a varying concentration of amine and acetamido groups respectively rather than for two discrete macromolecules. Thus it is necessary to find means of giving a detailed specification of the particular polymer being discussed.

Chitin is not an isolated compound in living organisms but exists within a predominantly protein and calcium carbonate matrix in animal sources with varying compositions in different species. Thus it is necessary to distinguish between the chitin as found in life forms and as it is used in the laboratory and industry. The latter can be considered as a degraded product of the first for which the term *native chitin* had been suggested (28). Depending on its origin, native chitin is complexed with various other biomolecules such as proteins and polysaccharides (fungal sources) to form systems of up to arbitrary complexity (like as sequences found in glycogonjugates). Pure native chitin is exceptional and has only been found in two diatom species.

Isolated chitin, already described in its idealised form as poly [β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose] is only obtained after purification under drastic alkaline and acidic conditions. With chitin referring to purified native chitin, the names chitin and chitosan describe a continuum of copolymers with different degrees of *N*-acetylation. With a decreasing degree of *N*-acetylation the polymer becomes more soluble in dilute acids due to the increasing amount of proton-accepting amine groups. Chitosan describes that range of copolymers soluble in dilute acids while chitin refers to the range of copolymers insoluble in dilute aqueous acid solutions. This method of specification is not the most logical or systematic in terms of chemical structure and subsequently has got some disadvantages in use. But having developed historically and bearing practical advantages it is still the one most commonly in use.

A different system would be to use the name chitin for the whole polymeric continuum giving the mole fraction of *N*-acetyl groups in brackets. With this method ideal chitin is called chitin[1.0] while idealised chitosan is chitin[0.0] and a commercial chitosan with a degree of de-*N*-acetylation of 75 % (*N*-acetylation of 25 %) is chitin[0.25].

The latter system seems to be the more desirable one for chemists but could lead to confusion e.g. amongst biologists who found different properties distinguishing chitin from chitosan.

Combining criteria, solubility and degree of *N*-acetylation, a more practical system for all parties concerned with chitin/chitosan is obtained. The mole fraction is still given in brackets. This turns chitin[0.25] and chitin[0.0], which are both soluble in dilute acid solutions into chitosan[0.25] and chitosan[0.0]. This system has been applied in this thesis, it can also be applied for *N*-acyl or other derivatives. Thus for example a completely *N*-hexanoylated sample of chitosan[0.01] can be described as *N*-hexanoylchitosan[0.01/0.99] adding the mole fraction of the other substituents after the acetyl group in order of increasing molecular weight (29).

Additionally although the random- or block-type nature of *N*-acetylation in differently produced chitosans has not been conclusively clarified (see Section 1.5.2.3 and 3.3.2.2), the observed different behaviour of heterogeneously as opposed to homogeneously produced materials (30) strongly suggests a significant difference in structure. Hence an index for sample history, while the nature of distribution is not yet completely clarified, would be useful. For cases where the nature of the distribution is known it was suggested that italicised letters could be placed after the number indicating mol fraction with r for random, b for block and a for alternating (29), e.g. chitosan[0.02b/0.33r].

1.2 Applications

1.2.1. Overview

A variety of factors in our modern world are encouraging the search for innovative solutions to current problems. The feasibility of a sustainable biomass-based chemical industry has been discussed favourably (31) in view of higher world population and increasing living standard. Factors such as depletion of traditional material resources like fossil carbon sources, conventional methods becoming obsolete like antibiotics losing their effectiveness, as well as economic and ecological issues, are challenging modern science and engineering to turn elsewhere. The increase in a world population that consumes more and more food, energy, pharmaceuticals etc., while at the same time causing pollution to soar, is increasingly raising issues like better crop protection at lower bio-toxicity and, improved efficiency of preventative and curative medicines, to name just a few, all at preferably low energy requirement and little pollution. The use of renewable plant and

Table 1.1: Biomedical applications reported for chitin, chitosan and related products

Application	Material	Source	References
Suppression of tumour growth	Chitin and chitosan derivatives		(32)
Inhibition of metastasis	Chitosan derivatives	Queen Crab	(33)
Immunotherapy	Chitin and chitosan derivatives		(32, 34)
Antithrombogenic	Chitosan	Queen Crab	(33)
Wound healing	Chitin and chitosan derivatives		(35)
Skin substitute	Polyelectrolyte complexes between chitosan and chitosan derivative		(36)
Periodontal tissue	Chitosan ascorbate		(37)
Healing of cartilaginous injuries	Glucosamine hydrochloride	Crab chitin	(38)
Orthopaedic use	Chitin	Japanese pink crab	(39)
Absorbable sutures	Chitin	Japanese pink crab	(40)
Arteriosclerosis	Chitosan		(41)
Drug carrier	Chitin and chitosan derivatives		(42-46)
Mechanical drug aid	Chitin and chitosan		(47)
Food poisoning	MYCOTON (chitin, glucans and melanins)	Fungi (Higher Basidiomycetes)	(48)
Side effects of chemotherapy			
Effects of radiation doses			
Antiallergenic			

Application	Material	Source	References
Side effects of chemotherapy	Chitosan		(49)
Bovine mastitis treatment	Chitosan in combination with antibiotics		(50)
Obesity	Chitosan		(49)
Inflammatory pain	Chitin and chitosan		(51)
Ophthalmology	Chitin, chitosan and derivatives		(52)

animal sources for innovative products as well as ensuring optimum use of resources and disposal of residual material has opened up the wide field of biotechnology. Complex carbohydrates, because of their versatility, are playing a primary role in this race for new solutions.

With the abundance of material available, the varied properties the polysaccharides chitin and chitosan exhibit and the number of scientists and engineers globally researching chitin, chitosan and derived materials, it is not surprising that a wide field of possible applications have been proposed and put into practice. While its antimicrobial and bioactivity as well as its low toxicity and biodegradability have opened up an ever increasing spectrum of important biomedical applications such as wound healing and cancer treatment, the copolymer's polycationic nature and viscosity have made it interesting for the food, cosmetic, paper, textile and other industries. Tables 1.1 and 1.2 have been compiled to give an overview of the variety of applications first in the biomedical field and second in general applications, while separation processes are considered in the next section.

Table 1.2: Various applications reported for chitosan and related products

Application	Material	Source	References
Textile printing	Chitosan	Commercial (medium molecular weight)	(53)
Anti-felting of wool	Chitosan and derivatives	Commercial	(54)
Higher mechanical strength in paper	Microcrystalline chitosan		(55)
Higher mechanical strength in paper	Chitosan	Commercial	(56)
Shoot and root growth	Chitosan		(57)
Seed treatment	Chitosan	Crab chitin	(58)
Growth and flower production	Chitosan		(59)
Cut flower quality	Chitosan	Cuttlefish	(60)
Preservation of fresh fruit and vegetables	Chitosan		(61, 62)
Preservation of tofu	Chitosan		(63)
Bio-fungicide	Chitin and chitosan as substrate		(64, 65)
Dry shampoo	Chitin		(66)
Hair styling	Hydagen® CMF		(67)
Cosmetic skin care			
Cosmetic skin care	MYCOTON (chitin, glucans and melanins)	Fungi (Higher Basidiomycetes)	(48)

1.2.2. Chitosan and its derivatives in separation processes

Chitin and its more reactive derivative chitosan are perfectly suited for separation purposes such as effluent treatment and many others. This is due to its unique properties as a low toxicity polycation with excellent film forming ability and a global abundance as a sustainable, biodegradable biomaterial second only to cellulose. Thus these polysaccharides can form the basis of adsorbents and flocculents and act as the structural polymer of separation membranes. This means that this versatile material can be employed as a package for various steps in separation processes, from highly concentrated to very low concentrations of solute or particle. With rising population numbers and increasing industrialisation globally, the depletion of natural resources such as fossil energy and carbon sources as well as inorganic materials, and the increase of solid and fluid (gases and liquids) waste volumes, are greater than ever. These give rise to a call for the use of renewable materials, and also separation and recovery of materials from solid and fluid mixtures employing low energy processes*. Chitin-based material as absorbents, flocculation agents and membrane components could be the answer to all of these questions. It is a renewable resource with an estimated annual production of $> 10^9$ t (8) and the second most abundant biomaterial after cellulose on this planet. Chitin, its more reactive derivative chitosan and some of their derivatives respectively are polycations in dilute acid medium and excellent flocculants and adsorbents for a number of species (see Table 1.3). While flocculation is more efficient than adsorption (69) adsorption has got the advantage that the adsorbents can theoretically be regenerated (70) and reused, while the flocculant often has to be disposed of with the species adsorbed. Thus adsorption is the method of choice especially when the species can be recovered, unless the coagulum can be utilised, e.g. as animal feed (71), then flocculation is favourable. Chitin, chitosan and their derivatives have also got excellent film forming abilities. (72) Their use has been reported for a number of separation processes (see Table 1.4). Flocculation/coagulation, adsorption and membrane separations are typically carried out at ambient temperatures and thus are processes with favourably small energy requirements. (73)

* 40% of the overall energy demand in the chemical processing industry is consumed by separation processes, which consists of distillation in 95% of the cases. (68)

Table 1.3: Separation of various species from aqueous solution with chitin, chitosan and their derivatives

Species	Effect	Polymer	Reference
Dyes	Adsorption	Chitosan	(4, 74-82)
		Chitin	(65, 68, 69)
		Derivatives	(70, 81, 83)
	Flocculation, coagulation	Chitosan	(84)
Heavy metals and radionuclides	Adsorption	Derivatives	(85)
		Chitin	(86)
		Chitosan	(87-101)
		Fungal chitin	(101)
		Fungal chitosan	(102, 103)
		Derivatives	(87, 92, 93, 95, 98, 99, 104-106)
Precious metal ions	Adsorption	Chitosan	(104, 107, 108)
Halogen			(109)
Suspended solids from food processing wastes	Coagulation, flocculation		(110)
Amino acids	Adsorption	Derivatives	(111)
Proteins from food processing waste	Coagulation, flocculation	Chitosan	(112)
Proteins from cheese whey			(10, 110, 112)
Suspended solids from municipal wastewater			(112, 113)
COD of wastewater			(99, 114, 115)
COD of wastewater	Coagulation, flocculation	Derivatives	(99)
BOD of wastewater		Chitosan	(115)
TOC of wastewater			(69)

Species	Effect	Polymer	Reference
Organic compounds in freshwater	Adsorption	Derivative	(53, 103, 104)
Bacteria in wastewater	Adsorption, flocculation	Chitosan	(112, 114, 115)
Bacteria in freshwater	Coagulation, flocculation	Modified chitosan	(116, 117)
Algae freshwater		Modified chitosan	(112, 116)
Fibre		Chitosan	(115)

Membrane separation is omnipresent in every living cell. This concept can only assert itself in nature as it is very effective and works efficiently at low energy requirements. Unsurprisingly mankind has tried to copy this idea for a long time. From the 1860s scientists like Fick and Schuhmacher started to work with the first artificial membranes made from collodion (=cellulose based polymers) (118). A lot has happened in the attempt to produce membranes for separation processes since then. Many separation problems can be solved with satisfying results, but yet there is still a lot of space for research on developing membranes that are equally effective as those present in nature. An obvious choice for copying a natural phenomenon would be to use materials already provided by nature, which is shown by cellulose being to date the most important starting material for industrial membrane production (73). When it comes to chemical modification however chitosan has got superior qualities compared to cellulose, since it can be crosslinked more readily and substituted more selectively. Thus its hydrophilicity as well as functionality are open to more specific and aim-orientated manipulation than those of cellulose. With chitosan's excellent film forming abilities these are ideal preconditions for developing tailor- made separation membranes. From the 1980s chitosan has been generating an increased interest among researchers as a starting material for a number of membrane separation processes (see Table 1.4).

Table 1.4: Membrane separation processes with chitin, chitosan and their derivatives

Species	Process	Polymer	References
Salts	Dialysis	Chitosan, *N*-acyl-chitosans	(72, 119, 120)
		Crosslinked chitosan (disulfide-bond)	(72)
Organic salts		Chitosan, *N*-acyl-chitosans	(119, 120)
Amino acids			(119)
Protein	Ultra filtration	Chitosan	(10)
Phosphate	Dialysis		(121)
Uric acid			(121)
Urea			(72, 121, 122)
		Chitin	(123)
		Crosslinked chitosan (disulfide-bond)	(72)
		Crosslinked chitosan (glutaraldehyde) protein coated	(122)
	Ultra filtration		(124)
Creatinine	Dialysis	Chitin	(123)
		Chitosan	(121)
Sucrose		Chitin	(123)
		Chitosan, Crosslinked chitosan	(72)
Glucose		Chitin	(123)
		Chitosan	(121)
		Crosslinked chitosan (glutaraldehyde) with protein coating	(122)
	Ultra filtration		(124)
Saccharose	Dialysis		(122)
	Ultra filtration		(124)
Vitamin B_{12}	Dialysis	Chitin	(123)
Cytochrome C	Dialysis		(123)
Dextran	Ultra filtration	Chitosan	(125)

Species	Process	Polymer	References
H_2O from pyridine	Pervaporation	Crosslinked chitosan (glutaraldehyde)	(126)
H_2O from EtOH		Crosslinked chitosan	(127)
		Crosslinked chitosan (glutaraldehyde)	(127)
		Crosslinked chitosan (H_2SO_4)	(128)
H_2O from ethylene glycol		Chitosan	(68)
MeOH, EtOH	Osmosis	Crosslinked chitosan (glutaraldehyde) with protein coating	(122)
	Ultra filtration		(124)
Glycerol	Dialysis		(122)
	Ultra filtration		(124)
Ethylene glycol	Dialysis		(122)
	Ultra filtration		(124)
Polyethylene glycol (400 - 4000)	Dialysis		(122)
Polyethylene glycol (400 - 6000)	Ultra filtration		(124)

1.3 Manipulation of properties in chitosan in respect to film formation

1.3.1 Influence of substitution on film forming and hydrophilicity

1.3.1.1 General

Several parameters have been isolated which affect the performance of chitin/chitosan in membrane applications. One of the major demands on a membrane is the selectivity for solutes that are allowed to pass through the membrane during separation. The permselectivity, that is the permeability for solutes of different sizes and charges, is dependent on the swellability and thus on the amount of free water contained in a membrane during quasi-stationary working conditions. The less swollen the membrane is, the higher is its permselectivity (119). The free water content of a chitosan-based

membrane can be manipulated by changing parameters such as hydrophilicity by introducing hydrophobic side groups (119, 120). Increasing concentration of hydrophobic groups results in higher organo-solubility as well as higher permselectivity. Further parameters to influence swelling ability are crosslinking (126, 127) and crystallinity. The swelling ability decreases with the increase in crosslinks formed and with increasing crystallinity (30). This means, the more amorphous the material, the more water it can adsorb and the bigger the pores of the membrane. For example the increase in polymer molecular weight causes a decrease in crystallinity (e.g. for *O*-butyrylchitin membranes (129)). Controlling hydrophilicity and crosslinking has got another important mechanical function. Depending on the fluid media of the separation process, the membrane needs to have specific solvent tolerances against structural disintegration. Chitosan membranes, for example, cannot be employed in acid medium unless they possess sufficient hydrophobic substituents to prevent solubility or are sufficiently crosslinked and thus protected from dissolution.

Hydrophobicity is not only an important parameter for membrane applications, but also for the production of fibres (130) and properties like shrink proofing where the introduction of long chain *N*-acyl groups improved the antifelting treatment for wool compared to that of chitosan (54). Another important characteristic of organo-soluble derivatives of chitin and chitosan is their comparatively high concentration in saturated solutions of up to 22% for di-*O*-butyrylchitin in acetone (130).

1.3.1.2 Preparation of organosoluble N- and O-acyl derivatives

Structurally very similar to chitosan, cellulose has been acylated to degrees of substitution up to 3.0, which means that all three free reactive groups per monomeric unit, hydroxyl in this case were reacted. Cellulose becomes increasingly soluble in non-polar organic solvents such as acetone and chloroform with increasing acetylation (131).

Organosoluble acyl derivatives of chitosan (132) and chitin (130) have been reported. The advantage of employing chitosan as opposed to cellulose is its higher regioselectivity as the more reactive amine group can be reacted independently from hydroxyl groups, thus making more fine-tuned modifications for tailor-made materials possible. While acetyl groups already occupy the majority of chitin's "amine" sites, it still possesses the potential of introducing different acyl groups at the hydroxyl sites as for example butyryl groups. These are larger than acetate group but butyryl anhydride is still comparatively small and

reacts with chitin to yield soluble products with perchloric acid as catalyst (7). The products were spun into fibres (130), cast into films to determine their physicochemical properties for biomedical applications (129) and coagulated as microspheres (7). The reason why chitin and chitosan have not provided as much potential for derivatisation as cellulose is that they are less accessible due to higher crystallinity (see also Section 1.1.2). High degrees of interaction between acetamido's oxygen and near-neighbour hydroxyl groups in intra- as well as intermolecular hydrogen bonding are increasingly an issue in materials with increasing *N*-acetyl concentration most pronouncedly seen in chitin's resistance to dissolution (see also Section 1.4.1). The same interactions have to be considered for materials with *N*-acyl groups other than acetyl in decreasing solubility (5), although bulkier substituents can be expected to induce steric hindrance to chain-chain interaction. The latter is supported by the increasing *O*-acetylation obtained for chitosan *N*-acyl-chitosan films, as monitored by infrared spectroscopy, with increasing linear aliphatic acyl group length, the biggest improvement being observed in between *N*-propionyl- and *N*-butyrylchitosan (133, 134). A comparision of X-ray diffraction patterns showed well defined rings for re-*N*-acetylated chitosan (reconstituted chitin) and increasingly diffuse patterns with increase in the length of the linear aliphatic *N*-acyl groups, indicating decreasing crystallinity in aggreement with increasing accessibility for *O*-acylation (134).

Selective *N*-acylation has been carried out under comparatively mild conditions in acetic acid/methanol systems 1:4-5 under addition of 2-3 mol carboxylic anhydride/mol monomeric unit at room temperature to yield derivatives of alkanic acids up to C10 and several other carboxylic acids. The degree of substitution was reported as [1.00] up to *N*-hexanoyl chitosan and >[0.80] for higher molecular weight substituents. The absence of ester groups was confirmed by IR spectroscopy (135). It has been shown that the methanol concentration in the system can be decreased to a ratio of 1:1 for aqueous acetic acid over methanol without nominal loss of reaction efficiency (6).

Direct *O*-butyrylation of chitin to yield an acetone soluble product has been carried out with a proportion of approximately 10 mol of anhydride per mol reactive site and an optimum amount of 0.5g perchloric acid (70%) per g chitin in an exothermic reaction. In order to retain the degree of polymerisation it has been found crucial to keep the temperature of the reaction system low at 20°C, while the effect of amount of catalyst was

found to be negligible in the range of 0.24g to 0.6g per g chitin (7). A direct method for *O*-acylation subsequent to *N*-acylation of chitosan was reported to yield products soluble in dimethyl sulphoxide and formic acid (132). In the same publication an indirect non-degradative route for the preparation of di-*O*-acetyl-*N*-acetylchitosan (chitin diacetate) soluble in dimethyl sulphoxide and formic acid was claimed. *O*-acetylation was carried out subsequent to protection of the amine group by Schiff's base formation with salicylaldehyde and benzylaldehyde. Removal of the protection group was achieved by mild acid hydrolysis and fully *N*-acetylated di-O-acetylchitosan was claimed. The still limited solubility, only two solvents being found, was explained by the high degree of polymerisation of the material being approximately 700 as opposed to 250 for high-grade cellulose triacetate.

1.3.2 Blending polymers as a means to materials with new properties

Membranes can be produced from polymer blends. These membranes can be heterogeneous, when the respective polymers do not intermingle, or inter-polymer membranes, when the polymer chains are homogeneously mixed with each other and form an inter-polymer network. (73) The obvious advantage of blending polymers is to combine favourable qualities of each into a membrane, which exhibits the desired properties of the involved materials and may show novel properties. The qualities of a polymer can be of a chemical nature, such as for example reactive sites as in the case of chitosan and its amine groups, or mechanical such as tensile strength, or even commercial such as low cost of a material. Membranes from chitosan blends with superior properties to the unblended and commercially available membranes have been reported (121). Here improved hydrophilicity and pore structure due to blending chitosan with poly(vinyl pyrrolidone) lead to a higher water content and improved permeability for low molecular weight metabolites such as urea, creatinine, glucose as well as uric acid and phosphates. Similar results were obtained by blending chitosan with poly(ethylene oxide) (136). The equilibrium hydration and thus porosity was increased due to intermolecular association between the two materials, which led to higher permeability for urea, creatinine and glucose compared to unblended chitosan membranes. Other properties such as platelet adhesion were favourably decreased. Selective transport phenomena of chloride ions though crosslinked chitosan-poly (vinyl alcohol) blend membranes have been studied (137) with pH difference as a driving force. Blends from polyelectrolytes of opposite charge can form polyelectrolyte complex membranes, which give rise to the possibility of controlling

the flux of solutes by changing the properties of the amphoteric membrane by changing local conditions such as pH. They have been suggested for protein separation (138). Chitosan with its unique cationic nature is of special interest. A disadvantage of polyelectrolyte complex membranes is their lack of homogeneity due to the blend's tendency to coacervate, precipitate or to form hydrogels of varying texture. (139) However, polyelectrolyte complex membranes exhibit a much higher hydrophilicity than the unblended membranes. It is obvious, that by employing polymer blends, the qualities of several materials can be combined into one membrane, which may additionally show novel properties.

Polyelectrolyte complexes can be prepared by blending chitosan with a range of anionic polymers, either other polysaccharides such as alginate or caragheenans or synthetic polyelectrolytes such as poly (sodium acrylate). Polyelectrolyte complexes between chitin and carboxymethyl cellulose have been reported and studied (138, 139). Sulphonated chitosan/chitosan complexes have been considered as a skin substitute (36). Some patents for chitosan/alginate mixtures have been published (140, 141) however no homogeneous solution of the two polymers such as may be formed using CHMS* (142) could be claimed.

A solution to overcome inhomogeneity in chitosan polyelectrolyte complex membranes caused by rapid complex-formation pre-empting homogenous blending has been patented (142). In this process chitosan is solubilised by formation of a complex with formaldehyde sodium bisulfite rendering it negatively charged. It has been possible to prepare homogeneous blends with a variety of anionic polymers such as alginate and sodium carboxymethyl cellulose to name just a few. The polyelectrolyte complex is not formed on blending but only subsequently by recovery of the chitosan, by destruction of the solubilising chitosan complex by acid or alkali. This opens up a wide field of new possibilities in chitosan blending and polyelectrolyte complex membranes. Membranes can be prepared by blending chitosans with varying F_A dissolved using the new solubilising process, with alginate, carboxymethyl cellulose and caragheenans (or any other polyanion) solutions. The blending can be undertaken at various proportions, concentrations, temperatures and pH values, since these casting conditions can impact strongly on the properties of polyelectrolyte membranes (139). The polymer blend

* CHMS: Sodium *N*-methylsulphonated chitosan

solutions can be cast onto a support and the solvent evaporated. In order to make the membranes resistant to chemicals as well as for pore size control the consideration of crosslinking with agents such as glutaraldehyde, citric acid and itaconic acid is useful. The general possibility of converting the blend chitosan to chitin or derivatives opens up further immense potential for creating materials with desirable properties.

Permeability and permselectivity depend largely on the swellability of a membrane. Varying the degree of deacetylation of chitin beween 100 and 80% (heterogeneous preparation of chitosan), which equates to a F_A range between [0.00] and [0.20] has been found to influence the swellability of a chitosan film inversely (143). Comparing materials prepared by re-*N*-acetylating chitosan of different initial F_A to the same degree of *N*-acetylation, it was shown that the crystallinity along with swellability of chitosan film was not only dependent on the end F_A, but also on the initial F_A. Hence, it was proposed, that crystallinity/swellability also depend on the substituent distribution in the final products. One material with low starting F_A was compared to a second with moderate initial F_A, both with comparable initial crystallinity as determined by X-ray diffraction. While the latter's crystallinity remained essentially constant the former's underwent a much more pronounced decrease (30). The content of free water in a polyelectrolyte complex is dependent on the pH of the casting solution, the ionic strength of the medium that the membrane is exposed to and the molar proportions of the polyions (139). Changing the pH of the ambient fluid can control the chemical and physical properties of the membrane, hence the selectivity and flux for solutes (138).

1.4 Solution properties of chitin and chitosan

1.4.1 Solubility of invertebrate chitin

Despite its structural similarity, crustacean chitin is not soluble in typical cellulose solvents like cuprammonium hydroxide. Chitin is very resistant to dissolution, especially when non-degraded or under non-degradative conditions due to its intractable crystalline structure and intra- and intermolecular hydrogen bonding.

Strong mineral acids dissolve chitin, but only in the course of severe decrease of molecular weight. In the search for non-degradative solvents for chitin out of 200 mixtures the two amide systems *N*, *N*-dimethylacetamide (DMAc)-LiCl and *N*-methyl-2-pyrrolidone (NMP)-LiCl have been found to be good solvent mixtures, the components themselves

being only swelling agents for chitin (144). More recently a new non-harmful solvent system to replace harmful chitin solvents namely saturated $CaCl_2 \bullet 2H_2O$ (>82% w/v) in methanol has been proposed and employed for viscometric studies of the molecular weight of chitin and chitin derivatives (145).
Further solvent systems have been proposed and found useful by some but of limited solubility by other workers. The use of solvents such as carboxylic and mineral acids, hydradative salts like e.g. LiCNS and $Ca(CNS)_2$, as well as solvents like DMF (dimethyl formamide) /N_2O_4 mixtures, which have been found to dissolve chitin to small extents (<1 g/l) has been comprehensively reviewed (12).

1.4.2 Solubility of chitosan

Despite its crystallinity chitosan is soluble in aqueous systems, as the amine group in chitosan can be protonated

$$-NH_2 + H_3O^+ \rightleftharpoons -NH_3^+ + H_2O$$

and thus acts like a base in the process of dissolution under acid conditions. At a pH <4.5 chitosan can be considered fully protonated (146, 147). The pK_a of chitosan is strongly dependent of the degree of *N*-acetylation (148). This solubility however comes at a price, namely acid hydrolysis which has been found to be about three orders of magnitude more pronounced for the glycosidic linkage between *N*-acetylated monomers as compared to that between amine monomers (149, 150). This makes highly de-*N*-acetylated material less susceptible than moderately *N*-acetylated polymer or even chitin. Chitosan is typically insoluble in water due to its crystallinity, which is confirmed by the observation that medium molecular weight chitosans of 1.6 to $3.1*10^5$ [0.01] to [0.37] tolerate the addition of NaOH without precipitating in the pH range of 5 to 8. The solubility was greater with higher F_A (149).

Chitosan's acidity is not constant, but depends on the charge density of the polymer and hence is a function of the degree of dissociation. It is also dependent on the degree of *N*-acetylation, however converging to a value of $pK_a \sim 6.5$ (=pK_0, a F_A independend constant) for the fully dissociated polybase (148).

Chitosan as a base forms salts with acids. The solubility of these salts determines the solubility of chitosan in the respective acid at different concentrations.
Thus chitosan is soluble in most dilute organic and inorganic acid solutions, but precipitates from higher concentrations of HCl (151) and HBr (152). The stability of chitosan salts stored in dry powder form as required for pharmaceutical and biomedical applications has recently been investigated (153). An exception is H_2SO_4, which does not dissolve chitosan in dilute but only in concentrated solutions however in the course of this, there is extensive hydrolysis of the polymer chain (154).

1.4.3 Properties and conformation of chitosan copolymer in dilute solution

1.4.3.1 Polyelectrolytes in dilute solution

Dilute solution viscosity can be related to the dimensions of the coil and thus the conformation of the polymer chain and its segments. Polyelectrolytes behave somewhat differently from uncharged polymers. While dilute solution viscosity such as reduced specific viscosity

$$\eta_{red} = \frac{\eta_{sp}}{c}$$

c =concentration

η_{sp} = specific viscosity

$$\eta_{sp} = \frac{\eta - \eta_s}{\eta_s} \approx \frac{t - t_s}{t_s}$$

η = viscosity of the polymer solution

η_s = viscosity of the solvent

t = drain time of the polymer solution (determined in a capillary viscometer)

t_s = drain time of the solvent (determined in a capillary viscometer)

decreases linearly for uncharged polymer with increasing dilution, for polyelectrolytes the viscosity increases in the area of very low polymer concentration with further dilution. The latter is a consequence of electrostatic repulsion of the charged segments and increased Stern layer thickness with thinning counterion concentration in dilute salt-free solution and

osmotic pressure induced expansion of the polymer coil. The polymer coil of a charged polymer chain occupies a larger volume than it would if the chain was uncharged. Thus it is necessary it is necessary to add a small amount of electrolyte to the solvent in order to ensure that the plots of reduced specific viscosity against polymer concentration are linear. The limiting viscosity number, derived from the zero polymer concentration intercept of the above plot, can then be used to determine the polymer's volume average molecular weight (see also Section 1.5.3.3c)). The addition of small amounts of low molecular weight electrolyte and thus the increase of ionic strength reduce the thickness of the Stern layer and the osmotic pressure and leads to contraction of the polymer coil and reduction of viscosity (155).

1.4.3.2 Chitosan copolymer in dilute solution

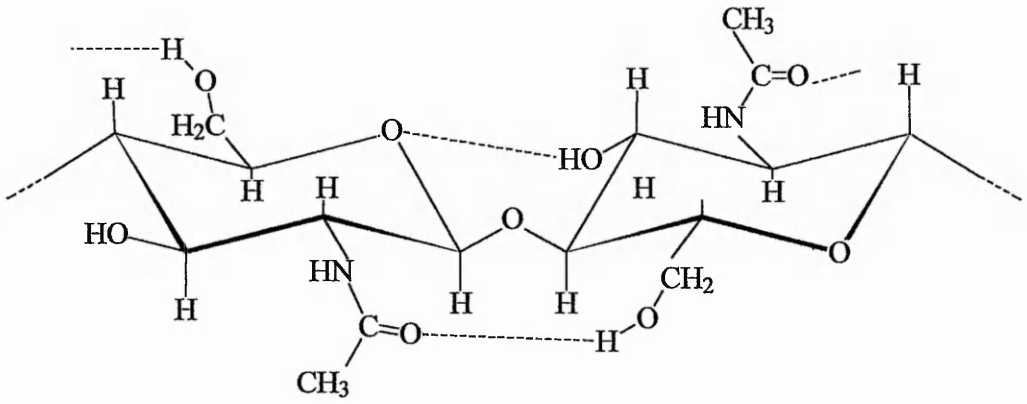
The dilute solution viscosity of chitosan is influenced by three main factors. Firstly chitosan is a polymer, which exists in solution as a random coil. Secondly chitosan is a polyelectrolyte with subsequent intra-molecular electrostatic repulsion in part responsible for the dimensions of the coil and an increase in reduced specific viscosity at low polymer concentrations (10^{-3}-10^{-2} g/L) observed at acetic acid concentrations of 0.17-1.7 *10^{-3}M (156).

In dilute acid solutions in the absence of added electrolyte, the viscosity number increases when the concentration decreases, as the coils inflate, because of the electrostatic repulsion which is then less hindered by neighbouring coils or by screening by the electrolyte ions (see previous section). In moderately concentrated solutions or in the presence of low-molecular-weight electrolytes chitosan shows the typical behaviour of a polymer, as these electrolytes screen the electrical charges (157) and "salt out" the polyelectrolyte character of the polymer. Here the viscosity increases with increasing polymer concentration and decreasing temperature. The limiting viscosity number decreases with increasing salt concentration (157) indicating a decrease in Stern layer thickness, which eventually leads to coagulation of the polymer in high salt concentration.

The viscosity also decreases the less protonated the polymer is, as the coil extension decreases with less repulsion. Excess acid itself has been found to decrease limiting viscosity number of chitosan with increasing concentration, hence to act like a low molecular weight electrolyte at low chitosan concentrations, however not as efficiently (158). Comparing the values of radii of gyration for a chitosan[0.12] obtained at different

values of ionic strength suggested that the degree of folding of the polymer chain is proportionally to the ionic strength of the solution (159).
The third important influence on the copolymer's dilute solution viscosity is specific to chitin/chitosan, namely its ability to increasingly form intra-molecular hydrogen bonds with increasing number of acetyl groups and thus carboxyl oxygen atoms (160) lately confirmed by statistical mechanical calculations of stiffness allowing for near-neighbour hydrogen bond formation (149).

Figure 1.3: Possible hydrogen bonding in chitin and chitosan (160)



This effect and possible steric hindrance due to the more bulky *N*-acetyl group* account for higher rigidity (143, 162) of the polymer chain even at lower charge density and thus electrostatic repulsion due to a decrease in rotational freedom around the glycosidic oxygen linkage. Molecular modelling for the influence of degree of acetylation from 0%-100% on the intrinsic persistence length as a measure for local chain stiffness has been carried out for chitin and chitosan and it was concluded that the chitosan chain is slightly more flexible than chitin with the persistence length varying from 90-125 Å at 50°C. However measurements of intrinsic viscosity were smaller than theoretically calculated, which implies discrepancies for the modelled and the true persistence length (163).

The commonly found experimental evidence is that the limiting viscosity number decreases proportionally with F_A, despite the evidence for increasing chain stiffness. This

* This has been suggested as a possible effect (160), however alginate as a similarly stiff molecule was found to retain its flexibility on acetylation and to accommodate acetyl-groups without restriction of rotational freedom (161).

phenomenon has been explained by the chitosan coil becoming more compact and thus hydrodynamic volume decreasing as chain repulsion decreases but the polymer changing shape from a random spherical coil to a flatter disc shape conformation (164). Chitosans of two different F_A values were compared by Wales-Van Holde ratio of sedimentation coefficient concentration regression coefficient over limiting viscosity number (chitosan[0.11]) and by use of the α value, which is related to the conformation of the polymer in dilute solution, in the Haug triangle (chitosan[0.42]) in a 0.2M acetate buffer at pH 4.3. It was proposed that the lower de-*N*-acetylated chitosan was present in a conformation with comparatively high rod shape contribution while the higher de-*N*-acetylated chitosan held a shape similar to a random coil (162). In order to separate the influence of electrostatic repulsion and isolate the influence of the *N*-acetyl group on chain conformation, α values calculated from measurements extrapolated to infinite ionic strength, at which the electrostatic interaction of the chain segments can be assumed suppressed, have been compared for chitosans ranging from [0] to [0.6]. Independent of ionic strength α values were found higher at higher F_A and it was concluded that the presence of *N*-acetyl group dominates the hydrodynamic behaviour of chitosan (165).

The limiting viscosity number, as would be expected, has been found to decrease with increasing ionic strength for chitosan[0.20] (157) and chitosans of F_A [0], [0.15] and [0.6] (165). As a measure of stiffness, convenient because of being independent from degree of polymerisation and its determination independently of molecular weight measurement, the empirical stiffness parameter *B* has been introduced (161). *B* is derived from a polyelectrolyte's tolerance and response to changes in ionic strength, which are more pronounced the more flexible the polymer chain is and are reflected in the changes of limiting viscosity number. The value of *B* is inversely related to stiffness of the polymer chain and has been found to be between 0.02 and 0.10 for chitosans [0.6] to [0] again confirming proportional relationship of stiffness and degree of *N*-acetylation. As a requirement for the validity of *B* it was also shown that chitosan does not undergo any conformational transitions within NaCl concentrations of 0.131M to 1.007M (165).

1.5 Characterisation of chitin and chitosan

1.5.1 General

Since chitin and chitosan are natural products they are also subject to natural variations as well as variations due to differing species, processing and preparation conditions, storage (microbial degradation) and further refining. For reproducible material properties and quality it is imperative to characterise and define the species employed and specify the materials according to their purity, degree of *N*-acetylation, molecular weight and for some applications, their crystallinity, particle size and sterility. Further parameters that are unconsidered, like humidity regain may become important in the future as understanding increases and novel chitin materials and applications are developed and commercialised. A first long awaited systematic approach for the overall characterisation and quality assessment has been published (166) for chitosan as well as a suggested standard (167) for pharmaceutical and biomedical application and very recently the first standard by the ASTM for biomedical applications of chitosan (168). Analytical determination in food has been suggested (169) as well as quantitation of nanomole amounts of chitin and chitosan by ELISA (enzyme linked immunoassay) (170). In the following the characterisation of chitosan by its molecular composition, degree of *N*-acetylation and molecular weight (distribution) shall be the main focus.

1.5.2 Degree of *N*-acetylation

1.5.2.1 General

Important for its concentration of reactive sites, solubility and biodegradability the degree of *N*-acetylation has been most frequently determined and a manifold range of methods have been used and comprehensively reviewed (12) p86-102. The acetyl group content or inversely the amine group content has been successfully determined by wet chemical methods. Hydrolysis and determination of released acetic acid assessed the acetyl group, while acid-base and colloid titration, dye interaction, consumption of periodate on oxidation of α-aminoalcohol in de-*N*-acetylated residues and salicylaldehyde consumption of the amine group determined the amine groups present. Instrumental methods such as IR, UV and NMR spectroscopy as well as circular dichroism have provided valuable

information on *N*-acetyl/amine substitution. Further methods like e.g elemental analysis and pyrolysis techniques have been investigated however to little avail.

1.5.2.2 IR

Since first proposed as a means of the determination of degree of acetylation (133), IR has been a popular method for chitosan as well as chitin. The technique does not require solubility of the material since samples can be prepared as KBr disks or films. The instrumentation is standard laboratory equipment and widely accessible to many researchers. Thus it is not surprising that a multitude of publications have dealt with the optimisation of this method. A number of researchers have used many different characteristic or probe bands changing with degree of acetylation and reference bands required to be independent of degree of acetylation relating the ratios to chitosan samples prepared in various ways and with independently determined F_A values.
A comprehensive statistical study has been carried out to evaluate the suitability of reported IR methods confirming some published bands A_{1561}/A_{1074}, A_{1561}/A_{1025} and proposing a few further ratios of characteristic bands over reference bands A_{1626}/A_{2877}, $(A_{1663}+A_{1626})/A_{2877}$ with their respective baselines for reliable results (171). The group also proposed optimised conditions such as absence of protein, minerals, H_2O and general purity (172). The latter discussion concluded that calibration curves and equations need to be established for chitosans specific to their preparation conditions. Another group however obtained satisfactory results for chitosans regardless of different sources using A_{1320}/A_{1420} band ratios at the respective baselines obtaining a calibration curve 0.3822 + 0.03133 * D_A (173). Both groups used ^{13}C CP/MAS* NMR spectroscopy as an independent method of analysis.

1.5.2.3 NMR (nuclear magnetic resonance) studies on degree and distribution of acetyl groups

^1H NMR spectroscopy as a method to determine the acetyl group concentration in chitosan was first used in the process of studying *N*-acetylchitosan gels by establishing the ratio of *N*-acetyl protons over methine and methylene protons in DCOOD (174). ^1H NMR was performed in DCl and CD_3COOD systems and compared to results from elemental analysis

* Cross polarisation magic angle spinning

and colloid titration with NMR and elemental analysis results being in better agreement with each other than colloid titration to the two other methods (175).

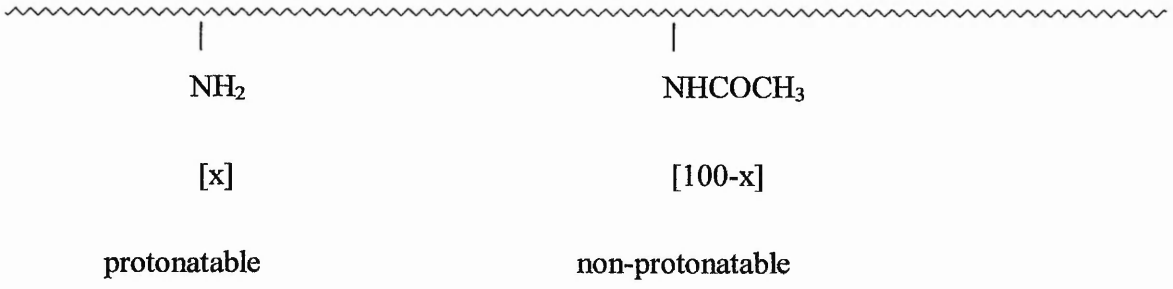
The concentration of *N*-acetyl groups was determined as well as the sequence of copolymer units in chitosan investigated with NMR spectroscopy the latter by looking at the sequence dependent de-shielding of the proton in the acetyl group of the different possible diads and determining their proportions. The results of heterogeneously prepared chitosan showed a proportion for the all *N*-acetylglucosamine diad slightly above random distribution, while homogeneously de-*N*-acetylated materials with the same degree of *N*-acetylation showed greater congruence with random distribution (176). A subsequent publication discussing triad sequences of comonomers by ^{13}C NMR concluded that a difference in substituent distribution between heterogeneously and homogeneously deacetylated chitosan could not be found (177). However the group themselves state that the accuracy of the triad frequencies is comparatively low (177). Furthermore it has to be asked whether the degradation carried out prior to analysis may have led to data loss and blurring of sequence information as well as the randomness of the degradation at higher concentrations of anhydro-*N*-acetyl-D-glucosamine monomers, since the latter are not susceptible to nitrous acid degradation. A further criticism of the investigation (164) is that clearly for some of the results (178, 179) (for others it is not clearly stated (176, 177)) the insoluble fractions, predicted most likely to contain block anhydro-*N*-acetyl-D-glucosamine monomers, of heterogeneously prepared material were removed from the NMR sample. Furthermore no explanation for different bulk properties found in homogeneous re-*N*-acetylated and heterogeneous de-*N*-acetylated chitosans at comparable substituent concentrations was offered. The insoluble residue was described as a "chitin like material" and suggested responsible for erroneously interpreted X-ray patterns as homopolymeric blocks (149). Comparison of homogeneously re-*N*-acetylated material of low initial F_A chitosan with the homogeneously de-*N*-acetylated material to the same acetyl group concentrations could provide further information and clarification.

1.5.2.4 Dye interaction

Dye interaction methods for the determination of the degree of *N*-acetylation have been successfully used as a low cost method with little equipment requirements, while at the same time yielding reproducible results. The general principle is to determine the EW of the protonatable group, which is the free amine group in chitin or chitosan at the pH range

regarded. The method mostly used in this investigation was dye adsorption with Acid Orange 7 (3, 4) since it can be used for chitosan, as well as chitin and gives reproducible results by stochiometric adsorption of anionic dye onto the cationic polymer sites at low operator times. The dye concentration with and without polymer at equilibrium is determined by UV-VIS spectroscopy and the difference related to the number of ionic sites per weight chitin/chitosan. The method was adapted for the determination of the level of substitution for chitosans with protonatable and non-protonatable secondary substituents (see Results and Discussion). The general principle of derivation of EW is elucidated regarding Figure 1.3, which gives a schematic idea of the substitution pattern in chitin and chitosan.

Figure 1.3: Schema of the substitution conditions along a chitin and chitosan chain respectively



The equivalent weight can be defined as the weight of 100 sugar units over the number of protonatable groups in 100 sugar units.

$$EW = \frac{x*161+(100-x)*203}{x}$$

$$\Rightarrow x = \frac{20300}{EW+42}$$

$$D_A = 100 - x$$

$$D_A = \frac{EW-161}{EW+42}*100$$

$$\Rightarrow F_A = \frac{EW - 161}{EW + 42}$$

EW = equivalent weight of amine group

x = percentage of anhydro-D-glucosamine units

D_A = degree of *N*-acetylation

F_A = fraction of *N*-acetylated sugar units

For detailed derivation and examples see appendix.

A second method to obtain EW by dye interaction is metachromatic titration. In this method the absorbance is plotted against increasing volume of chitosan solution added to a series of dye-containing flasks. The graph yields an equivalence point (EP) at which the dye concentration equals the concentration of ionic polymer sites (see also sections Experimental and Results and Discussion). From the latter the EW can be determined. Metachromasy and metachromatic titration generally have been comprehensively reviewed (180, 181) and metachromatic induction by chitosan has been reported (182-185). Thus chitosan as the acid soluble copolymer has been determined with Acid Orange 7. The method, like dye adsorption, gives reproducible results as confirmed by IR spectrometry (3) and requires little equipment. However, unlike the dye adsorption method, considerable operator time and acid solubility of the polymer are required.

1.5.2.5 Further methods

Reflecting the importance of this parameter a wide variety of feasible wet chemical and instrumental methods has been proposed for analysing the amine group or inversely the acetyl group concentration in chitin and chitosan. Additional instrumental methods suggested are elemental analysis (186), UV-VIS spectroscopy (187, 188) and gas chromatography (189). Apart from metachromatic titration as described in Section 1.8.2.4, titration methods like colloid titration proposed for polymer ions generally and also chitosan (190, 191) and acid-base titration methods (151, 152) have been reported. As further UV-VIS spectroscopy related techniques, the interaction with picric acid has been investigated (6, 80, 192) as well as the quantitation of residual salicylaldehyde after quantitative Schiff's base derivatisation of the amine group with excess of aldehyde (152).

1.5.3 Molecular weight and its distribution

1.5.3.1 General

Next to degree of *N*-acetylation the molecular weight (typically meaning average molecular weight in the following) and its distribution or polydispersity (weight average molecular weight over number average molecular weight) are crucial determining factors for chitin and chitosan's properties, as for polymers in general. While researchers usually specify the degree of *N*-acetylation of a material, the molecular weight let alone molecular weight distribution often is not mentioned. This is due to the fact that a facile, economic and at the same time reproducible method has not been satisfactorily established. Highly sophisticated methods such as SEC-MALLS and triple detector SEC have been successfully used to determine chitosan molecular weights and distribution patterns (193, 194) but have the disadvantage that only a comparatively small number of laboratories are equipped with the necessary instrumentation. Various scientists have considered viscometry, as a suitable technique for the determination of molecular weight with low equipment requirements. A recent review however of published k and α values (195) has shown that the Mark-Houwink relationships for chitosan molecular weight could not be reproducibly clarified yet and further investigation of the method is necessary.

In the following an overview of various methods for determination of molecular weight and molecular weight distribution will be given with a focus on chitosan, since the analysis for chitin has posed major problems due to the material's lack of solubility in simple solvents.

1.5.3.2 Instrumental methods with SEC as fractionation tool

a) General

Aqueous size exclusion chromatography (SEC) also known as gel permeation chromatography (GPC) has been used for the purification of proteins and nucleic acids as well as the estimation of protein molecular masses and the determination of molecular mass distribution of polysaccharides. Otherwise very similar to HPLC (high performance liquid chromatography), the separation principle in SEC, as its name already suggests, is based on the size of molecules and thus due to steric effects between the solute and the

chromatography support only. The latter is typically negatively charged, which poses a challenge to optimise the experimental conditions such as electrolyte choice and concentration in the aqueous eluent and the addition of acetonitrile to suppress hydrophobic attraction with aromatic side groups. Interaction with the support needs to be suppressed by comparatively high salt concentrations especially when the eluted species are like chitosan positively charged (196).

b) SEC with secondary calibration by dextran standards

For estimation of molecular sizes in chitosan by an absolute method size exclusion chromatography calibrated with dextran standards has been reported as a satisfacory method, SEC data relating closer to flocculation performance than viscometric data (197). Assuming equal calibration factors for chitosan and dextran, justified by their structural similarity, optimum conditions like column combination, load and polymer concentration for the analysis of chitosans with this method have been claimed (198). Molecular weight determination of chitosan by SEC has been reviewed and compared to SEC-MALLS analysis (193). It was found that the slope of calibration curves of log weight average molecular weight (MW_w) *versus* polymer elution volume for dextrans was greater than that of chitosans. Thus error occurs due to the greater hydrodynamic volume of chitosan under these conditions, causing the latter to elute earlier than dextran at the same molecular weight. Considering that chitosan will possess different charge concentrations due to different degrees of acetylation present it does not seem feasible to find conditions under which a standard like dextran would exhibit the same hydrodynamic volume as the chitosan analysed. This discrepancy and further error due to insufficient suppression of polymer/support interaction may have led to some curiously constant results for the development of polydispersity over time of progressing random alkaline deacetylation of chitin. The polydispersity was reported to only slightly increase at values above 4.5, rather than decreasing and approaching the theoretic value of random distribution of 2.0 as would be expected for random chain degradation by 50% during the same reaction time (197).

c) Multidetector SEC

Using SEC as a fractionating tool to get the molecular mass distribution has involved applying various detection methods such as refractive index and different light scattering techniques to yield weight average molecular weight and dilute solution viscosity to obtain viscosity average molecular weight. In addition to obtaining molecular weight information

the methods also have been employed to gain a deeper understanding of the conformation of different chitosans in dilute solutions.

When studying the molecular dimensions by light scattering an accurate determination of the specific refractive index increment dn/dc is of great importance. Various researchers have determined dn/dc values in the process of light scattering experiments.

Table 1.5: Specific refractive index increment for chitosan solutions reported in literature (in chronological order)

F_A	dn/dc /[mL/g]	λ/[nm]	Solvent	Reference
[0.11] ±[0.01]	0.166		Not stated	(159)
Not stated	0.174 (average value)	436, 546	8.5% formic acid/0.5M sodium formate, 25°C	(186)
[0.132] to [0.212]	0.160	632.8	1% HOAc/0.2M NaOAc	(199)
[0.42]	0.180 ±0.006	633	0.1M HOAc/ 0.2M NaCl, 25°C	(200)
[0.28]	0.183 ±0.005			
"low" (<<[0.28])	0.201 ±0.006			
[0.01] to [0.24]	0.181	632.8	0.333M HOAc/ 0.1M NaOAc	(193)
[0.31], [0.16], [0.09], [0.0]	0.175, 0.189, 0.194, 0.208	436	0.2M HOAc/ 0.1M NaCl, 25°C	(160)
[0.02], [0.09], [0.17], [0.29]	0.181, 0.176, 0.169, 0.157	633	0.3M HOAc/0.2M NaOAc, RT	(158)
	0.193, 0.186, 0.176, 0.167		0.2M HOAc/0.1M NaOAc, RT	
[0.12] to [0.61]	0.190 ±0.005	Not stated	0.3M HOAc/0.2M NaOAc, 25°C	(163)

Table 1.5 shows that various scientists have assumed that dn/dc is independent of F_A with good approximation while another two studies independently found that there is an

increase in dn/dc with decrease in F_A (160, 200). A source of seemingly too high molecular weights is the aggregation of polymer chains in solution, especially with decrease in the solvent's ionic strength (159). Preheating the polymer solutions has proven a good measure to counteract aggregation over time and in addition minimised standing times (148, 201) and electrolyte solvent systems such as 0.3M HOAc/0.2M NaOAc have been proposed to reduce the presence of aggregates (147). The dependence of dn/dc on the ionic content of the solution is thought to be negligible (201). For improved structural information the use of triple detection systems subsequent to SEC fractionation coupling refractive index, light scattering and viscometric detectors has been studied. Triple-detector analysis has got the advantage of providing more structural data such as viscometric information about polymer folding as well as molecular weight distribution* per sample preparation and sample amount (194). Recently multidetector SEC has been suggested for the determination of the molecular weight of chitin in DMAc-LiCl and *O*-butyrylated chitins (202).

1.5.3.3 Capillary viscometry

a) General

Viscometry, through determination of the limiting viscosity number (LVN/[mL/g], also called intrinsic viscosity/[dL/g]) presents a useful method for monitoring possible relative chain degradation of a specific material during processing and refining.

$$LVN = \lim_{c \to 0}\left(\frac{\eta_{sp}}{c}\right) = \lim_{c \to 0}\left(\frac{\ln \eta_{rel}}{c}\right)$$

$$\eta_{rel} = \frac{\eta}{\eta_s}$$

(See also Section 1.4.3.1)

* and the degree of branching for non-linear polymers

The intrinsic viscosity is a measure of the hydrodynamic volume of a polymer in a specific solvent system and at a discrete temperature, assuming infinite dilution and thus non-interaction with further polymer molecules. Through the Mark-Houwink relationships

$$LVN = k * MW^{\alpha}$$

$$MW = \sqrt[\alpha]{\frac{LVN}{k}}$$

the average viscosity molecular weight can be related to the limiting viscosity number of a polymer by empirical determination of the k and α values or functions specific for a polymeric material. The value of α is influenced amongst others by the chain flexibility and is predicted to increase with increase in stiffness or persistence length (155). For the validity of the equation it is required that the polymer is unbranched and of narrow molecular weight distribution, the latter also needs to be of similar shape for samples and reference samples.
Various researchers have investigated the potential of using viscometry as a convenient method for characterising chitosan's molecular weight and various constants have been claimed to be valid for chitosan in diverse conditions such as solvent composition and temperature.

Practice shows, that molecular weight determined by viscometry does not necessarily correlate to chitosan's properties as found to be the case for molecular weight determined by an absolute method (SEC) (197). Discrepancies have been found such as the molecular weight determined by light scattering varying up to 50% for different chitosan samples, while the intrinsic viscosity remained nearly constant (159). Various researchers (158, 195, 203, 204) have evaluated viscometric constants of chitosan for the Mark-Houwink equation with even the most recent work concluding that no satisfactory relationship for this material has been found yet and that further work is necessary.

b) Chitin

The determination of molecular weight of chitin itself has posed major problems due to its lack of non-degradative solubility (see also Section 1.4.1) other than in very harmful complex solvents such as e.g. DMF-N_2O_4 and DMAc-LiCl. Recently a new non-harmful

solvent system, saturated $CaCl_2 \cdot 2H_2O$ (>82% w/v) methanol systems and its potential for the determination of chitin molecular weight by viscometry has been used to propose tentative k and α values for chitin (2.54, 0.45 respectively) (145).

c) Chitosan

Extensive work has been done on the determination of suitable solvent systems and constants for the viscometric determination of chitosan's molecular weight. Some work has been done without considering the degree of acetylation, while others have found this parameter to be of negligible importance for the hydrodynamic volume or the polymer in solution (163). Hydrodynamic volume as a measure of molecular dimensions is the crucial determining factor of dilute solution viscosity of a polymer. It is dependent on the extension of the polymer chain in solution and thus for a polyelectrolyte like chitosan largely influenced by its charge density and the resulting electrostatic repulsion of chain segments. The stiffness of the chain in chitosan is increased by the concentration of acetyl groups due to their intoduction of intra- chain hydrogen bonding (160) resulting in coil deformation. Intrinsic viscosity itself has been reported to increase with decreasing ionic strength but to be independent of the species of counterion (157). However another publication gives markedly different values for limiting viscosity number for chitosan in solutions of 0.2M NaCl and 0.2M NaBr namely 374 mL/g and 454 mL/g for the same chitosan sample (201). A likely explanation for this discrepancy is that in the former investigation the chitosan polyelectrolyte was already somewhat swamped with acetate ions by being dissolved in 0.7M HOAc. The further addition of salt carried out in concentrations between 0.05M and 0.5M then did not make a significant difference in the hydrodynamic volume of the polymer coil. Due to the polyelectrolyte nature of chitosan it is necessary to use solvent systems containing small electrolytes to compensate the charges on the polymer when determining dilute solution viscosities such as inherent and reduced viscosity and thus various systems have been suggested as can be seen in Table 1.6.
The most recent study concludes that the hydrodynamic volume is independent of the amine group concentration (163). However, when plotting the paper's tabulated k and α values against F_A a flat but very clear trend can be seen for both the heterogeneously de-*N*-acetylated material and to a slightly lesser extend the homogeneously re-*N*-acetylated chitosan, with k decreasing and α increasing proportionally with F_A. The latter indicates that the rigidity of chitosan chains in solution increases with increase in F_A due to the

presence of intra-chain hydrogen bonds between the carbonyl oxygen as well as the heterocyclic oxygen and hydroxyl groups in the vicinity. The bonding restricts the rotation of the monomers around the glycosidic link. This restriction is diminished with progressing deacetylation as the amine group, which is protonated in solution, is unable to form similar bonds with hydroxyl groups. The k values decreases with increasing F_A as a consequence of increasing polymer expansion due to the electrostatic repulsion being proportional to the charge density, which pushes the chain segments apart so opening the coil structure (160).

Table 1.6: Constants and conditions proposed for use of the Mark-Houwink equation for determination of molecular weight in chitosan

Solvent system/T	**F_A range**	**Range of MW/[10^5]**	**k/[mL/g], α**	**MW determined by**	**Ref.**
F_A not taken into consideration					
0.2M HOAc/ 0.1M NaCl/ 4M urea, 25°C	-	1.13-4.92	$8.93*10^{-2}$ 0.71	Sedimentation and diffusion	(205)
1% HOAc/ 2.76% NaCl, 25°C	[0]-[0.10]	0.12-1.7	1.115 0.147	Sedimentation and diffusion	(206)
0.1M HOAc/ 0.2M NaCl, 25°C	-	0.90-11.4	$1.81*10^{-3}$ 0.93	End group analysis	(207)
0.33M HOAc/ 0.2M NaOAc, 25°C	[0.15] ±[0.03]	0.61-1.6	$1.38*10^{-2}$ 0.85	Sedimentation and diffusion	In (158)
0.33M HOAc/ 0.3M NaCl, 25°C	-	0.13-1.35	$3.41*10^{-3}$ 1.02	-	In (158)
1% HOAc, 30°C	-	2.05-6.57	$4.74*10^{-2}$ 0.723	-	(208)

Solvent system/T	F_A range	Range of MW/[10^5]	k/[mL/g], α	MW determined by	Ref.
F_A considered					
0.1M HOAc/ 0.2M NaCl, 25°C	[0.40]-[0]*	-	$1.81\text{-}10^{-3}$ 0.93	-	(203)
0.2M HOAc/ 0.1M NaOAc, 30°C	[0.31] [0.16] [0.09] ~[0]	1.94-25	$0.104*10^{-3}$, 1.12 $1.424*10^{-3}$, 0.96 $6.589*10^{-3}$, 0.88 $16.80*10^{-3}$, 0.81	Light scattering	(160)
Chitosan chloride salt in H_2O/0.1M ionic strength, 20°C	[0.60] [0.15] ~[0]	0.15-1.64 0.35-2.45 0.15-3.10	$2.18*10^{-3}$, 1.06 $5.85*10^{-2}$, 0.78 $5.59*10^{-1}$, 0.58	Osmometry	(165)
0.3M HOAc/ 0.2M NaOAc, 25°C	[0.21] [0.115] [0.02]	-	$7.4*10^{-2}$, 0.76 $7.6*10^{-2}$, 0.76 $8.2*10^{-2}$, 0.76	SEC/MALLS	(147)
0.2M acetate buffer pH=4.3, 25°C	[0.42]		α=1.14	Sedimentation and diffusion	(162)
0.3M HOAc/ 0.2M NaOAc, 25°C	[0.25] to [0.005]	0.6-2.8	$6.8*10^{-2}$, 0.800 to $8.0*10^{-2}$, 0.796	SEC/MALLS	(163)
0.3M HOAc/ 0.2M NaOAc, 25°C	[0.61] to [0.02]†	0.75-1.35	$5.6*10^{-2}$, 0.821 to $7.9*10^{-2}$, 0.796		

The variety of proposals suggests a limited overall validity and has been evaluated to some extent. The reasons for discrepancies are most probably to be found in the use of highly polydisperse material and inaccuracies in the determination of molecular weight by an

* Materials prepared by homogeneous re-*N*-acetylation
† Materials prepared by homogeneous re-*N*-acetylation

independent method. SEC methods for example may give inaccurate molecular weights due to adsoption of the cationic material onto the column.
It has been concluded that there is still work necessary to obtain valid and accurate constants and conditions under which chitosans molecular weight can be determined reproducibly by viscometry (195) over its range of charge densities and molecular weight distributions, and which takes in the influence of polydispersity.

1.6 Main objectives of this project

Establishing relationships between the structure and the properties of species raises two main issues: the ability to selectively modify and the ability to assess the modification achieved as well as the overall configuration of the material before and after modification. Thus the investigation of the modification of chitosan concerning its chemical composition as well as other property relevant parameters like degree of polymerisation and the ability to characterise the materials before and after modification alongside each other have been the focus of this work
Modifications of chitosan were undertaken for various reasons, such as the preparation of novel materials with favourable properties like solubility in one medium, insolubility in different media, solvent retention, homogenous miscibility in selected systems as well as creating standard species for analytical investigations. In the following a summary of the objectives of this study is given.

- Testing and preparing different pathways to chitosan-based materials for membranes such as

    - Formation of organo-soluble chitosan derivatives by introduction of hydrophobic substituents on amine groups and hydroxyl groups selectively
    - Crosslinking of chitosan with non-hazardous agents like citric and itaconic acid suitable for insolubilisation of formed in place membranes or other chitosan morphologies
    - Characterisation of a patented water soluble chitosan derivative (CHMS*) with negative charges for making homogeneous blend formation of chitosan with the

* CHMS: Sodium *N*-methylsulphonated chitosan

more varied polyanionic materials such as alginate , carboxy methyl cellulose, caragheenans and others possible

- Preparation of a novel film-forming derivative di-*O*-butyryl-*N*-hexanoylchitosan soluble in and film forming from simple and inexpensive organic solvents due to the hydrophobic substituents

- Characterisation of the degree of substitution of the patented chitosan derivative (CHMS)

- Preparation of a matrix of chitosan materials prepared from one sample of deacetylated high molecular weight chitosan with 3 discrete molecular weight distributions (low medium and high) and subsequent preparation from each molecular weight fraction of 5 samples varying only in degree of *N*-acetylation in the range of 15% to 65% but not their degree of polymerisation distribution

- Determination of the limiting viscosity number values for the above 15 chitosan matrix samples

- Preparation of substituted chitosan materials (CHMS) from portions of the above samples by a non-degradative process with the materials only varying from the initial chitosans in their chemical composition, but not in their backbone degree of polymerisation distribution

- Identification of a suitable solvent system for measurement of dilute viscosity for the samples of CHMS

- Determination of the limiting viscosity number values for the above CHMS samples

- Obtaining degree of polymerisation distribution data of key samples of the chitosan as well as the CHMS matrix determined by the absolute method SEC-MALLS in collaboration with LMPB (Laboratoire des Matériaux Polymères et des Biomatériaux), Prof. A. Domard, France for CHMS samples and Biopolymer Engineering Inc., Dr. G. R. Ostroff, USA for chitosan samples

- Comparing the data for degree of polymerisation distribution obtained for the corresponding samples of the matrices

- Evaluation of the reproducibility of degree of polymerisation distribution across the range of re-*N*-acetylation degrees

- Evaluation of the chain length retention during sodium *N*-methylsulphonation of chitosan

- Relating the limiting viscosity number and degree of polymerisation distribution data of the chitosans and corresponding CHMS samples to obtain new accurate relationships for the k and α values of the Mark-Houwink equation for estimation of molecular weight from the limiting viscosity number values for chitosan valid for a wide range of F_A from [0.148] to [0.609] and three different degree of polymerisation distributions

- Relating the limiting viscosity number and degree of polymerisation distribution data of the CHMS samples to obtain the first relationship for the k and α values for the new polymer

- Evaluating the potential of CHMS as a suitable material for degree of polymerisation distribution analysis by SEC separation methods due to its anionic/amphoteric character and thus less adsorptive nature compared to the cationic chitosan

- Formulate consequences for the architecture of chitosan and CHMS in dilute solutions following from degree of polymerisation distribution determination and dilute solution viscosity behaviour

2. Experimental Methods

2.1 Materials

2.1.1 General

Table 2.1: Chitosans employed

Name	Producer	Batch-reference	Viscosity	F_A	Abbre-viation
Chitosane MV	Aber Technologies	(Chit 98) Ref: A32E03 (06/07/95)	Medium viscosity, 65 cps (1% solution in 1% HOAc, RT)	[0.09]	CHa
PRO FLOC-P	Protan Laboratories Inc., USA	Lot No. 047-342-079	High viscosity, 230 cps (deacetylated to [0.15], purified, 1% in 0.1M HOAc, 17.5°C)	[0.28]	CHb
Black Tub	The Design of Materials Group	ARK/DE1	16 cps (1% in 0.1M HOAc, 20°C)	[0.28]	CHc

CHa was used unless otherwise stated.

General grade reagents and solvents were used as supplied.

Crude chitosan and the two dyes - C.I. Acid Orange 7 and a pre-purified sample of Methylene Blue (BB9) provided by the SDC - were purified according to the methods described in Section 2.2. A third dye C.I. Basic Red 2 (BR2) was used as commercially supplied.

Ballmilled *N*-hexanoyl chitosan (H) with a residual amine concentration of [0.02] was used as previously prepared (209).

2.1.2. Different morphologies of chitosan and its derivatives

2.1.2.1 Never-dried chitosan

Never dried chitosan was prepared by reprecipitating chitosan from acidic solution with methanolic ammonia as described in more detail in section 2.2.1. After neutralising the chitosan precipitate it was stored in MeOH until needed. For some applications chopping the material in a Waring commercial blender decreased the particle size for more facile heterogeneous reaction.

2.1.2.2 Films

Films of chitosan, its derivatives and blends for further reaction and analysis were produced by transferring amounts of the solution onto a clean* glass plate or micro slide. The latter had been placed onto a larger glass plate previously levelled with a spirit level. Film thickness was adjusted by levelling the liquid out with a doctor blade. The whole system then was covered to ensure dust protection while providing good ventilation at the same time to allow the solvent to evaporate at RT. The films were normally neutralised by steeping in methanolic ammonia followed by washing in distilled water.

2.2 Purification methods

2.2.1 Chitosan by reprecipitation

Purification was carried out by first dissolving the crude chitosan flakes over several hours of agitation in 0.1M HOAc to yield solutions of 2% for CHa and 2.7% for CHc and subsequent reprecipitation purified. CHc is more soluble due to a lower MW as indicated by its lower viscosity. The solution was then filtered through double layered polyester monofilament mesh in order to remove undissolved particles. Methanolic ammonia was added to the filtered solution on stirring to reprecipitate the polymer. When the precipitation was complete, at which point the coagulum visibly separated from the aqueous supernatant, no more ammonia was added. The precipitate was washed (distilled water) and filtered until neutral according to litmus paper, after which solvent was exchanged with MeOH. The solvent exchange makes it easier to manually break down the caked filter residue into small particles. These in turn were dried to a more openly

* Ensuring the absence of surface-active substances.

structured polymer with less inter-chain hydrogen bonding (see also WAN drying of cellulose (210)) at a temperature of about 40°C in a ventilated drying cabinet. The latter has been found to be more time-efficient than drying in a vacuum oven. Chitosan reprecipitated from solutions of higher concentration form a coagulum that is easier to process, that is to wash and to filter afterwards.

2.2.2 Dyes by recrystallisation

A 5% dispersion of the dye in 80% v/v EtOH was refluxed until dissolved. After cooling the solution down to zero reflux, the solution was filtered through glass wool into a conical flask, which was only loosely covered, and placed onto a cork ring for insulation and decelerated cooling for improved crystallisation. After standing for several hours the mixture was further cooled down in a refrigerator to encourage the growth of larger aggregates in the course of dissolution equilibrium, due to dissolution of smaller particles for the benefit of larger particles. The precipitate was filtered and washed with a small amount of 80% EtOH, after which it could be dried.

A further method employed for larger dye amounts of Acid Orange 7 was Soxhlet extraction of the dye with MeOH up to high precipitate values in the solvent fraction. The latter was then cooled down slowly on the hotplate and transferred to a refrigerator to improve crystallisation. The extinction coefficient of a typical, cloudy crude Acid Orange 7 concentration was determined to be 17426 L cm^{-1} mol^{-1}. The published extinction coefficient is 22500L $cm^{-1}mol^{-1}$ (211). It was found necessary to have a value >21000 L cm^{-1} mol^{-1}, and as close to the reported value as possible, in order to assure the absence of contaminants that would affect the dye-chitosan interaction equilibrium. Typical values of purified dye would be >21500 L cm^{-1} mol^{-1}. The exact dye concentration in analysis was determined by assuming the validity of the published value and the absorbance measured on a reference solution of the same lot that had not undergone polymer interaction, but that had otherwise received the same treatment.

2.2.3 KBr by reprecipitation

General reagent grade KBr was purified to a fine crystalline powder by preparing a saturated KBr solution in distilled H_2O and pouring the solution into acetone. The precipitate was filtered without standing time, since smaller crystals are easier to press to clear discs for IR spectometry. The filtrate was dried in a vacuum oven to constant weight.

2.3 Analytical methods

2.3.1 Viscometry

2.3.1.1 Determination of bulk viscosity

Viscometric measurements were carried out using a *Brookfield Synchro-Lectric LVF* viscometer.

Viscosity was measured by lowering cylindrical spindles of different geometries for different viscosity ranges into the bubble-free polymer solution. The cylinder was rotated at different speeds depending on the viscosity of the solution and the torque necessary to overcome the viscous resistance to the induced movement was measured. The values obtained were converted to viscosity in centepoises (cps) by using the conversion chart supplied.

2.3.1.2 Determination of dilute solution viscosities

The samples were dried to constant weight under vacuum and then stored in a desiccator with spot checks for any weight increase. For the dilution method samples of approximately 0.4g were accurately weighed into tared beakers and dissolved in approximately 50mL 0.1M HOAc overnight. To this was added 10mL of 2M solution of NaCl in 0.1M HOAc and the total solution weight was made up to 100g with 0.1M HOAc giving a solution having an overall solvent composition of 0.2M NaCl/0.1M HOAc. From this initial solution approximately 50g was accurately weighed into a volumetric flask and an equal weight of 0.2M NaCl/0.1M HOAc solution was accurately weighed into the same flask to obtain a solution of approximately 0.2g polymer/100mL after thorough mixing. From this solution samples with concentrations of 0.1g/100mL and 0.05g/100mL were produced in the same way. The exact concentrations of the solutions are calculated under consideration of the previously determined densities of the solvents employed. The solutions were kept in *a Townson & Mercer LTD* bridge control series III viscometer bath at 25°C in volumetric flasks so they could be introduced into the suspended level viscometer of Fitzsimmons type, modified with a glass sinter for filtration, also submersed at the appropriate temperature. For every new lot of 0.2M NaCl/0.1M HOAc solvent the run time t_0 was determined first. For every determination the viscometer was rinsed by pumping up the fluid twice, discarding the solution and filling the viscometer with new

solution. To keep inaccuracies to a minimum the solutions were measured from the lowest concentration (solvent) to the highest concentration (initial solution). The flow times between the two marks were taken using a *Heuer* microsplit 1000 stopwatch until three subsequent measurements agreed within 0.1% of each other (212).

2.3.2 UV/VIS Spectrometry

2.3.2.1 Instrumentation

Absorbance measurements for F_A and F_S analysis were carried out on a *Perkin Elmer 551S* UV/VIS spectrophotometer and a UV/VIS Spectrophotometer Unicam 8625. The former was also employed for transmittance measurements for the determination of turbidity in gels.

2.3.2.2 F_A/F_S analysis by dye adsorption of amine groups (3, 4)

A stock solution of $5*10^{-3}$M (1.75 g l^{-1}) purified Acid Orange 7 in 0.1M HOAc was prepared. Accurately weighed samples of chitin or highly substituted *N*-acylchitosans (approx. 0.2g) or chitosan (approx. 0.1g) were transferred into Quickfit conical flasks and aliquots of stock dye solution of 100mL for chitin and 200mL for chitosan were added accurately with a 50mL dispenser. For accurate determination of the concentration of the stock dye solution a control blank without polymer was prepared in the same way. The stoppered flasks were placed in a water bath and when the temperature had stabilised at 60°C the pressure was released and the flasks were sealed and left for 16h. After reaching equilibrium the solutions were swiftly filtered through glass wool, in order to remove the adsorbent and prevent diffuse adsorption on cooling, sealed and left to cool. After cooling the solutions including the control solution were diluted, typically 1:100, and the absorbance measured (λ_{max} =484nm in 0.1M HOAc, ε_{max}=22500 L cm^{-1} mol^{-1} (211)) against 0.1M HOAc as a reference.

The equivalent weight of protonatable groups such as the amine group in *N*-substituted /*N*-acetylated chitosans can be calculated by:

$$EW = \frac{w * \varepsilon \max}{\Delta A * f * V}$$

EW = equivalent weight of amine group /[g mol^{-1}]

w = oven dried weight of polymer /[g]

ε_{max} = extinction coefficient /[L cm^{-1} mol^{-1}]

ΔA = difference in absorbance values of blank dye solution and test dye solution

f = dilution factor (typically 100)

V = volume of dye aliquot /[L]

The mole fraction of *N*-acetylated glucosamine residues F_A in chitin and chitosan can be calculated by:

$$FA = \frac{EW - 161}{EW + 42}$$

In this study the F_A of chitosan was typically determined by dye adsorption.
For formulas for the calculation of substitution in other chitosan derivatives see section Results and Discussion.

2.3.2.3 F_A/F_S analysis by metachromatic titration with Acid Orange 7 (3)

A stock dye solution of $5*10^{-4}$M (0.175 g L^{-1}) purified Acid Orange 7 in 0.1M HOAc was prepared. A polymer solution was prepared by accurately dissolving 0.1g of the polymer in 1L of 0.1M HOAc (or higher amounts for derivatives with higher m_0). Ten 100mL volumetric flasks were prepared with 10mL aliquots of stock dye solution subsequently diluted with 40mL of 0.1M HOAc to minimise flocculation. After mixing, typically the following portions of polymer solution were added to the flasks: 0, 2.0, 4.0, 8.0, 10.0, 12.0, 15.0, 20.0 and 25.0, and the flasks were made up to 100mL with 0.1M HOAc. Absorbances of these solutions were measured (λ_{max} =484nm in 0.1M HOAc) and a graph of Absorbance *versus* Volume of chitosan solution was plotted. Extrapolating the graph's slope before and the (near) horizontal line after equivalence gives the equivalence point of amine groups and dye molecules. The intercept of both lines gave the amount of chitosan solution at the equivalence point. The EW can be calculated as follows:

$$EW = \frac{w * V}{10 * c}$$

EW = equivalent weight of amine group /[g mol^{-1}]

w = oven dried weight of polymer /[g]

V = volume of chitosan solution at the equivalence point /[mL]

c = concentration of the original stock dye solution /[mol L^{-1}]

(Determined accurately by absorbance, ε_{max}=22500 L cm^{-1} mol^{-1} (211))

For calculating the mole fraction of *N*-acetylated glucosamine residues F_A in chitin and chitosan see previous section, for substitution patterns of further derivatives see Results and Discussion.

2.3.2.4 F_S analysis by metachromatic titration with basic dyes

Stock dye solutions of the basic dye in distilled H_2O were prepared. A polymer solution was prepared by dissolving between 0.05g and 0.2g (accurately weighed) of the material in 250mL to 1000mL distilled H_2O depending on the anticipated EW of the material. Ten 100mL volumetric flasks were prepared as in section 2.3.2.3, but were made up to 100mL with distilled H_2O. Absorbances of these solutions were measured at λ_{max} and a graph of Absorbance *versus* Volume of chitosan solution was plotted.

2.3.3 Infrared Spectrometry

IR spectrometry was carried out on a *Jasco FT/IR-410* spectrometer.

Samples were prepared in the form of films, when a suitable solvent was available, or KBr discs for insoluble material. Films were produced by dissolving the compound at concentrations between 0.5% and 2% and casting a film onto a glass plate, which was placed onto a level surface and screened to protect the film from ambient dust. After evaporation the film was removed with a razor blade and further dried in a desiccator. For discs approx. 2mg of sample material were mixed with 200mg of KBr and ground in a *Specamill* agate ball mill. The powder mixture and the die parts were heated at 105°C in order to minimise H_2O adsorption. The die was assembled including the mixture, evacuated for several minutes and pressed at 8t for 10min. The spectrum of the clear disc was taken immediately.

2.3.4 Gravimetric determination of degree of substitution in sodium *N*-methylsulphonated chitosan

The degree of substitution of sodium *N*-methylsulphonated chitosan was determined by acid hydrolysis of the secondary substituent and gravimetric determination. Portions of approximately 0.5g or 1g of the material were accurately weighed into tared glass crucibles (+stirrers). The latter had been placed into small beakers allowing for overall immersion and magnetic stirrer agitation to take place in the crucible to prevent the particles from forming surface-area-decreasing agglomerates due to swelling on addition of 25mL 2N H_2SO_4 in aqueous, methanolic or ethanolic solution as the hydrolysing agent. After hydrolysis under agitation at RT for between 16h and 64h the material was neutralised with methanolic ammonia, washed exhaustively with 50% EtOH and dried. The weight of the crucible/stirrer/material was determined. Checking its swellability in H_2O tested the dried material for completion of conversion to chitosan.

2.3.5 Monitoring syneresis in gel systems

N-acylation as well as *N*-carbamylation induced gelation of the reaction systems. The gels showed syneresis, which was monitored in the following way. The weight of the initial system (polymer/solvent/reagent) was determined after mixing. After a period of time the exuded solvent was discarded and the gels were carefully padded dry with laboratory tissue and weighed. The weight loss and the time were recorded. The gels were not cut loose from the moulds as it has been shown (213) that the difference in syneresis of a loose acyl-chitosan gel compared to an attached gel, levels off in less than 24 h.

2.3.6 Determination of the average DP and DP distributions of chitosan and the corresponding CHMS samples by SEC-MALLS (size exclusion chromatography – multi angle laser light scattering)

Chitosan samples were analysed by SEC-MALLS in collaboration with Dr. G. R. Ostroff, Biopolymer Engineering, USA.

The CHMS samples were determined in collaboration with Prof. Alain Domard, Laboratoire des Materiaux Polymeres et des Biomateriaux, Université Claude Bernard, France by Jean-Michel Lucas.

Table 2.2 Parameters of the SEC-MALLS analysis

Conditions	Chitosan	CHMS
Solvent	0.333M HOAc/0.1M NaOAc buffer (pH 4.2)	0.5M FSBS buffered at pH 7
Filter	0.1μm	0.2μm
Column	TSK gel	TSK gel
Flow rate	0.6mL/min	0.5mL/min
dc/dn	0.181(193)	0.140
Detector	Wyatt-Dawn model F	Wyatt-Dawn DSP
Wavelength	632.8nm	632.8nm

2.4 Preparative Methods

2.4.1 Homogenous *N*-acylation(5, 6)

For the *N*-acylation of chitosan a 1% polymer solution in 0.1M acetic acid was diluted 1:1 with MeOH (plus varying amounts of co-solvent as stated) and the respective anhydride was added in molar proportions ranging from 0.8 to 6.0 based on free amine groups. Immediately before mixing the anhydride was diluted with part of the MeOH for better dispersion and easier dosage on addition to the reaction mixture. The mixtures were readily prepared with stirring in appropriate vessels or moulds and covered with laboratory film unless otherwise stated. Complete reaction was assumed after 16h. *N*-acetylation systems for syneresis studies were treated as described in section 2.3.4. *N*-acylated chitosan for preparative purposes was left to undergo syneresis at least over night, after which it was finely chopped in a *Waring* commercial blender, the solvent/impurities exchanged with MeOH and then dried in a vacuum oven at approximately 60°C. For partial re-*N*-acetylation of chitosan for materials matrix see section 2.4.9.

2.4.2 Heterogeneous *O*-acylation (7)

O-acylation was carried out with *N*-hexanoylchitosan. The ballmilled *N*-hexanoylchitosan powder was dispersed in different solvent/reagent/catalyst systems and reacted at room temperature under agitation for 16h.

2.4.3 Carbamylation

Chitosan solutions of varying concentrations in 0.1M HOAc were prepared and a freshly prepared solution of KCNO* in H_2O was added. Typically the mixtures would be agitated to homogeneity and then not further stirred. The molar proportions of KCNO/free amine groups ranged from 0.3 to 3.0. The end concentration of HOAc in the system was adjusted to 0.05M by the addition of H_2O. For some experiments MeOH as a co-solvent was added in varying proportions. Completion of reaction was assumed after 24h.

2.4.4 Crosslinking with itaconic acid and citric acid (di- and tri-carboxylic acid)

For crosslinking reactions the chitosan was dissolved in HOAc and solutions of the respective carboxylic acids were added on stirring. The concentrations of all three components were varied. The obtained solutions were cast onto glass plates supported by a level base, where they were left to evaporate at RT. After evaporation the films were treated at different temperatures in an oven and radiated with microwaves for different lengths of time. Immersing the plates in 0.1M HOAc tested the effect of crosslinking.

2.4.5 Deproteination of squid chitin

Deproteination of squid chitin was carried out on hammer milled native squid pen. The material was reacted in 1% (w/w) NaOH at 80°C for 1.5h (including heating time) in a water bath under occasional shaking and subsequently rinsed well to remove the brown-yellow foul-smelling protein denaturation products.
The protein content of the native squid pen material used was found to be 69%.

2.4.6 Decalcification of squid chitin

The deproteinated material was decalcified without further drying in 2M HCl under agitation at RT. The pH was monitored and found to stay low for the duration so that the process was concluded complete and the material washed to neutral according to litmus. The material loss due to decalcification was 3%.

* The KCNO concentration was adapted as suitable for respective dosage and dispersion.

2.4.7 De-*N*-acetylation of chitin and chitosan

2.4.7.1 De-N-acetylation of squid chitin

The dry chitin is mixed with the minimum amount of 45% NaOH (w/w) required. The aqueous NaOH solution is prepared first and left to cool, before adding to the chitin. The mixture is then heated at 55-60°C for 6 hours with air excluded. A test of the material for solubility in 1M HOAc was negative and the material was further reacted for 15.5h, which yielded a soluble but irreversibly yellow coloured product. This was processed by washing (H_2O) and filtering to neutral. The material had a F_A of [0.167]. Further de-*N*-acetylation to [0.156] was achieved by repeating the process with a reaction time of 6h. A different method was employed to obtain more drastic de-*N*-acetylation. The de-*N*-acetylation was carried out as described in 2.4.7.2, however on a hot plate under agitation at setting 9 (90°C) and addition of the whole amount of $NaBH_4$ at the beginning of the reaction. The resulting F_A was determined as [0.001].

2.4.7.2 De-N-acetylation of chitosan

De-*N*-acetylation of chitosan (specification see 2.1 c)) was carried out in 45 % w/w NaOH solution with a liquor ratio CH/caustic of 1:20 w/w. The polymer particles were dispersed in the NaOH solution and reacted in a sealed vessel placed in a water bath at 80°C for 1h. Addition of of 0.1g $NaBH_4$ (214) per 1g chitosan overall in 3 portions at the beginning of the process and after 20 and 40 minutes prevented oxidative chain degradation. Manual agitation was carried out coinciding with the addition times in order to keep the contact of the reaction mixture with oxygen to a minimum. After cooling down, the material was washed to neutral and dried. It was purified by reprecipitation as described in section 2.2.1 and its bulk viscosity was determined as 230cps (0.1M HOAc, 17.5°C).

2.4.8 Preparation of chitosans with different molecular weights by chain degradation with nitrous acid

Chitosans of different molecular weights were produced from one purified sample CHb by chain scission with nitrous acid. The high molecular weight chitosan was divided into thirds by weight. One third was kept as high molecular weight fraction and two thirds were dissolved to give a 1% solution in 0.1M HOAc. The dissolved material was divided into two lots. A moderate amount of 0.1mol $NaNO_2$/mol $-NH_2$ (215) was dissolved in 25mL of

H_2O and added to yield HNO_2 in the acidic reaction medium. Measuring the bulk viscosity with a *Brookfield Synchro-Lectric LVF* viscometer enabled the progress of the reaction to be monitored. The medium viscosity lot was reprecipitated with methanolic ammonia to stop the reaction and recover the material after the viscosity was decreased from 150 cps (22°C) to 75 cps and the low MW lot was reprecipitated after the viscosity had dropped from 100 cps (23°C) to 60 cps. After recovery and determination of LVN the latter material was further degraded, this time as a 2% as opposed to 1% polymer solution, from 69 cps to 34.5 cps (22.5°C).

2.4.9 Homogeneous partial re-*N*-acetylation of highly de-*N*-acetylated chitosan with reference to the special requirements for the F_A-MW chitosan matrix

Re-*N*-acetylation was carried out similarly to the procedure described in section 2.4.1. However in order to obtain a matrix of products with only 3 discreet values of MW and MW distribution and 5 discreet values of *N*-acetylation great care was taken to ensure reproducibility of reaction and process conditions. At any one time 3 samples, one portion of each molecular weight, were reacted to obtain one discreet value of the 5 different target degrees of *N*-acetylation reproducibly within the triplet. This ensured controlled processing within the limitations of instrumentation and person power at hand. Amounts were weighed out, even for liquids, for greater accuracy unless otherwise indicated. Solution preparation was carried out in a temperature-conditioned laboratory. Immersing the reaction vessels into a viscometer bath kept the solutions' temperature at an exactly reproducible level during homogeneous reaction. The subsequently precipitated materials were washed to neutral with MeOH and were recovered by centrifugation. The latter procedure and the exercise of great care minimised the overall loss of especially low MW material as well as general cross-contamination, maximising the accuracy of the envisaged LVN relationships. After achieving neutrality the material's overall wet weight was determined for later yield calculation and for dividing the material into two fractions. The first fraction was dried as chitosan for F_A and LVN analysis. The second fraction was stored as never dried CH in MeOH for later conversion to CHMS. The exact reaction times and conditions as well as system compositions can be found in the following Tables 2.3 and 2.4. Exceptions from the data in these tables are discussed in the Results and Discussion 3.3.2.1 e).

Table 2.3: Reaction times and conditions

Process	**Conditions/comments**	**Target time**
Dissolution of of chitosan in 0.1M HOAc (298.35g ~ 300mL, one lot for all 15 chitosan samples)	T=20.0 ± 2°C, humidity: 65% ± 2	17.5-18h
Dilution with (195.65g ~ 250mL) MeOH	RT (20-25°C)	10-20min
Mixing of reagent mix (7.15mL Ac_2O/250mL MeOH)	RT (20-25°C)	Up to 8 min
Dilution of reagent mixes (H, M, L) (amounts see next table) filled up to 40mL MeOH	RT (20-25°C)	6-20 min
Addition of the diluted mixes to the CH fractions, rinsing mix vessel with 10mL MeOH	RT (20-25°C), stirring vigorously for homogenisation	
Reaction	25.00± 0.01°C, immersed in viscometer bath	3.5h
Precipitation (end of reaction and recovery of CH)	RT (20-25°C)	
Addition of first washing solvent	RT (20-25°C)	After 30min
Centrifugation - washing for neutrality	36°C, 3 solvent exchanges, 30min spinning cycles	

Table 2.4: Reaction procedure for preparation of different fractions (in chronological order)

Target: F_A=[0.25]	**CH-H**	**CH-M**	**CH-L**
CH/[g]	2.0	2.0	2.0
0.1M HOAc/[mL]	200	200	200
0.1M HOAc/[g]	198.90	198.90	198.90
MeOH/[mL]	150	150	150
MeOH/[g]	117.39	117.39	117.39
Ac_2O/MeOH/[mL] + approx. MeOH	5.1 35	5.1 35	5.1 35
Target: F_A=[0.45]	**CH-H**	**CH-M**	**CH-L**
CH/[g]	2.0	2.0	2.0
0.1M HOAc/[mL]	200	200	200
0.1M HOAc/[g]	198.90	198.90	198.90
MeOH/[mL]	150	150	150
MeOH/[g]	117.39	117.39	117.39
Ac_2O/MeOH/[mL] + approx. MeOH	15.5 35	15.5 35	15.5 35
Target: F_A=[0.35]	**CH-H**	**CH-M**	**CH-L**
CH/[g]	3.0	3.0	3.0
0.1M HOAc/[mL]	300	300	300
0.1M HOAc/[g]	298.35	298.35	298.35
MeOH/[mL]	250	250	250
MeOH/[g]	195.65	195.65	195.65
Ac_2O/MeOH/[mL] + approx. MeOH	14.5 25	14.5 25	14.5 25
Target: F_A=[0.65]	**CH-H**	**CH-M**	**CH-L**
CH/[g]	3.0	3.0	3.0
0.1M HOAc/[mL]	300	300	300
0.1M HOAc/[g]	298.35	298.35	298.35
MeOH/[mL]	250	250	250
MeOH/[g]	195.65	195.65	195.65
Ac_2O/MeOH/[mL] + approx. MeOH	46.6 5	46.6 5	46.6 5
Initial: F_A=[0.15]	**CH-H**	**CH-M**	**CH-L**
CH/[g]	3.0	3.0	3.0
0.1M HOAc/[mL]	300	300	300
0.1M HOAc/[g]	298.35	298.35	298.35
MeOH/[mL]	250	250	250
MeOH/[g] overall	234.78	234.78	234.78
MeOH/[mL] without reagent	50	50	50
Ac_2O/MeOH/[mL]	-	-	-
	No reaction, simulation of reaction conditions		

2.4.10 Preparation of the water-soluble chitosan complex CHMS by sodium *N*-methylsulphonation with FSBS (formaldehyde sodium bisulfite)

2.4.10.1 Heterogeneous process

CHMS was prepared from the respective chitosan sample. The latter had been stored under non-degradative condition as never-dried chitosan. In the case where chain degradation is not an issue the never-dried chitosan had been chopped to decrease the particle size to improve diffusion of the reagent into the polymer. Reactions were carried out in systems of an overall solvent composition of 50 – 75% MeOH, EtOH or meths. When retaining the overall DP distribution is crucial, the reaction needs to be carried out in alcohol concentrations above 65%. The solvent volume ranged from 50 – 100mL per gram chitosan dry weight. The reagent FSBS previously dissolved in water was added in an amount of 2g/g chitosan dry weight. The mixture was stirred to improve reaction. Reaction time was a minimum of 19.5h. Taking a small sample from the reaction mixture, drying it and observing its solubility in H_2O tests for completion of reaction. After ensuring that the product was soluble the product was recovered by washing with 75% alcohol (MeOH, EtOH or meths). After exhaustive washing the solvent was exchanged with pure alcohol (and diethyl ether if quick or air-drying was envisaged). The solvent exchange ensured that the drying particles did not swell and agglomerate during drying, when alcohol evaporates faster leaving H_2O enriched solvent behind.

2.4.10.1 Homogenising reaction

The reaction of chitosan with FSBS in H_2O can be regarded as dissolution due to complex formation. Samples of chitosan of 0.5g and 0.3g were dispersed in 25mL of 4% FSBS in H_2O. The quickly swelling particles dissolved after 3.5h – 6h approximately. The solutions were poured into acetone with stirring to recover the polymer by reprecipitation. Steeping in 100mL 75% aqueous meths for 1h and decanting washed the precipitate. The latter was repeated 2 times (3 times overall) with aqueous meths and another time with pure meths for solvent exchange. The polymers were subsequently torn to smaller fibrous particles and dried to yield quickly dissolving materials.

3. Results and Discussion

3.1 Specific manipulation of hydrophobicity of chitosan by selective *N*- and *O*-derivatisation

3.1.1 Homogeneous *N*-acylation

3.1.1.1 Introduction

Chitosan can be selectively *N*-acylated under homogeneous conditions at RT with respective anhydrides in dilute HOAc/MeOH systems, while the absence of MeOH allows for some *O*-acylation to occur (5). With progressing substitution, the polymer hydrophilicity as well as inter chain electrostatic repulsion decrease due to a decrease in the concentration of protonated amine groups. Eventually the chains start to form aggregation areas aided by increasing chain stiffness with increasing F_A (149, 160), gelation sets in at a fraction of substitution of approximately [0.60] and firm clear gels can be observed at $F_S \approx [0.80]$ (213). Gels are characterised by their ability to contain large amounts of solvent, whilst exhibiting solid phase properties. However with the onset of gelation another phenomenon can be observed, namely syneresis. The gel contracts due to further aggregation of the polymer chains and exudes solvent; it undergoes syneresis. The relationship of molar proportions of free anhydride/amine and the reaction efficiency is linear 1:1 up to approx. $F_S=[0.5]$ (6) and then levels off due to steric effects and transport phenomena in gels. For complete substitutions a molar proportion of 1:3 based on free amine group content was employed (132).

The syneresis behaviour of *N*-acetyl gel (= regenerated chitin) systems was studied with varying parameters such as anhydride/amine group proportions, effect of ether as a co-solvent, head-space volume and molecular weight of the chitosan.

3.1.1.2 Homogeneous N-acetylation under various conditions

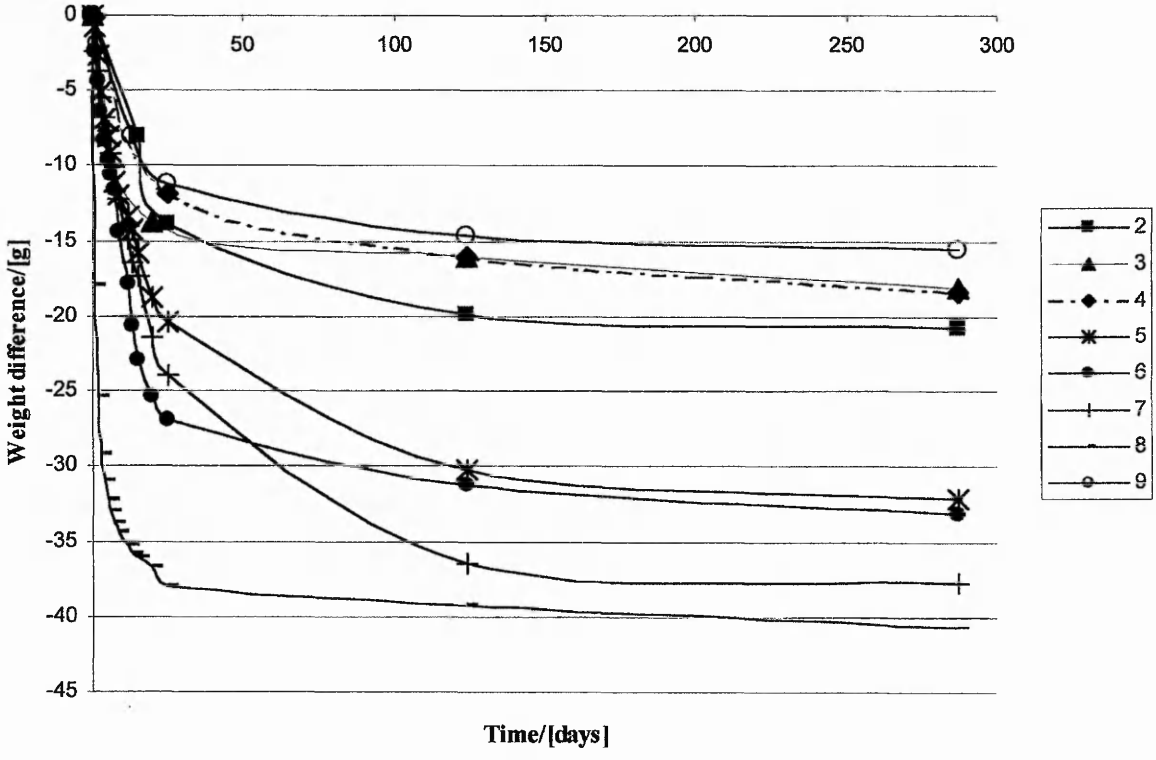
a) Effect of different anhydride/amine proportions

As can be seen in Table 3.1 the molar proportions of anhydride/amine groups were varied from 0 to 6.00 (samples 1 to 8). Sample 9 was monitored as a reference sample without the reagent anhydride, simulating the solvent/polymer composition and indicating the

contribution of solvent loss through evaporation to the overall solvent loss (evaporation + syneresis). The solvent system consisted of 0.1M HOAc/MeOH/ether with proportions of 3:2:1 parts by volume. Ether as a co-solvent was originally introduced in regard to acylation reactions with increasingly hydrophobic higher anhydrides, which become less soluble in the 50% aqueous MeOH system successfully employed with e.g. hexanoic anhydride.

The sample with the lowest anhydride content with a molar proportion of 0.85 did not show gelation in this solvent system, despite sufficient reagent for a F_A>[0.60]. This can be explained by the lower polarity of the solvent in the presence of ether. The partially *N*-acetylated polymer is obviously not hydrophobic enough to exhibit sufficient tendency for chain aggregation under the present less polar conditions. Gel systems of anhydride to amine ratios ≤2.05 were very stable. They lost less than 40% of solvent in a period of some 300 days, of which only ≈15% is due to syneresis as illustrated in Figure 3.1. The systems with ratios ≥2.37 however exhibited solvent loss in the range of approx. 60% to 70% overall, with 35% to 45% due to syneresis over the same period of time. Two influences

Figure 3.1: Range 0 - varying anhydride concentration



are effective here. On one side the increase in hydrophobicity of the polymer, and on the other increasing polarity of the solvent system due to an increase in acetic acid and anhydride, the latter forming the acetic acid in the reaction and by hydrolysis both favour increasing aggregation, gel contraction and syneresis.

Table 3.1: Composition of reaction systems and reagent amounts of series 0 and C and resulting degree of acetylation

Series 0 and C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Chitosan solution/[g]*	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
MeOH/[ml]	18.4	17.6	16.7	16.1	15.5	14.9	14.3	8.6	20
Ether/ml	10	10	10	10	10	10	10	10	10
Ac_2O/MeOH 1:10 / [ml]	1.6	2.4	3.3	3.9	4.5	5.1	5.7	11.4	-
Molar proportion Ac_20/-NH_2	0.84	1.26	1.74	2.05	2.37	2.68	3.00	6.00	0.00
Approx.(intended) proportion**	0.85	1.25	1.75	2.00	2.25	2.75	3.00	6.00	0.00
F_A for series 0		0.87	0.94	0.95	0.96	0.97	0.97	0.99	
F_A for series C			0.88	0.89	0.92	0.94	0.94	0.96	

* [Medium viscosity (series 0)/low viscosity (series C)] chitosan

** 0.85 was chosen, since formation of a firm gel starts at approx. 80% acylation (213)

** 6.00 was chosen, since it has been reported (216) that syneresis in chitin gels reaches an equilibrium at above ratio

b) Effect of co-solvent concentrations

The gels with ether as a co-solvent were clearer than comparable gels without ether, as the resulting polymers are more soluble in this solvent system and less inter-polymer connection points are likely to have formed. Ether/MeOH proportions ranged from 0.2 to 2.0, increasing the ether while decreasing the amount of MeOH. No gelation was observed at a ratio >1:1. A decrease in F_S could be observed with increasing ether concentration and decreasing MeOH concentration. This stresses again the importance of methanol (12) for the reaction, the efficiency of which decreases parallel with methanol concentration (6, 217). A sufficient F_A, hence sufficient hydrophobicity for gelation in this medium could not be achieved. Gels formed when using ether/MeOH proportions of 0.4 and 0.6 show

syneresis proportional to the amount of ether present, which can be explained by the higher volatility of ether (see also 3.1.2.3). The system with a ratio of 1:1 only exhibited slight gelation, which is due to a lower F_S. The gel was not as firm, which indicates lower cohesion forces within the gel and thus less contraction, which is reflected in lower solvent losses.

Figure 3.2: Range A – varying co-solvent ether concentration

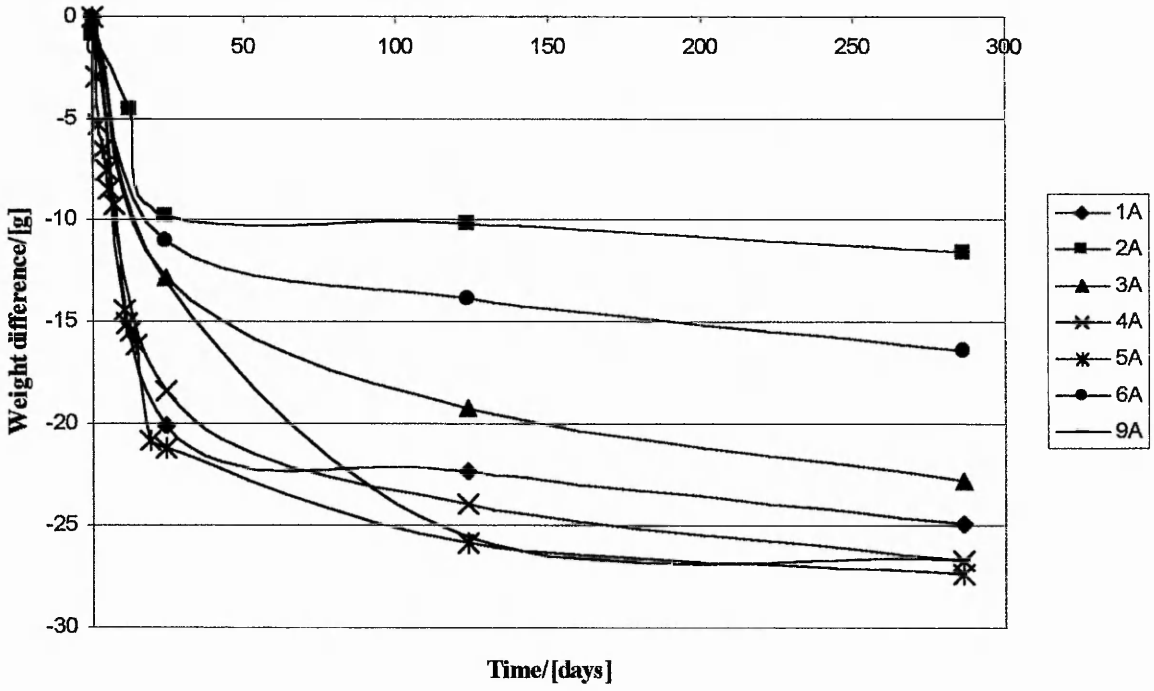


Table 3.2: Overview of series A: Composition of reaction systems and reagent amounts, resulting F_A and qualitative evaluation of gelation behaviour observed

Series A	1	2	3	4	5	6	7	8
Chitosan solution/[ml]	30	30	30	30	30	30	30	30
MeOH/[ml]	20.5	18.5	16.5	14.5	12.5	10.5	8.5	5.5
Ether/ml	5	7	9	11	13	15	17	20
Ratio ether/MeOH	1:5	1:3.3	1:2.3	1:1.7	1:1.3	1:1	1:0.8	1:0.5
Ac_2O/MeOH 1:10 / [ml]	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
F_A	0.94		0.95	0.96	0.92	0.88		
Gelation	+	-	+	+	+	(+)	-	-

\+ = Gelation occurred

\- = Gelation did not occur

(+) = Only slight gelation occurred

c) Effect of head-space volume

The head space volume of the systems was varied by applying different coverings to the moulds. Groups of gels were covered with *Parafilm* (laboratory film, ="pf", small head-space), cling film (="cf", large head-space) and left uncovered (="nc", infinite head-space). Obviously the weight loss was proportional to the volume of head-space. For gels with infinite head-space the amount of ether did not have a pronounced effect on solvent loss unlike for the gels with finite head-space, where increase in ether concentration clearly leads to higher solvent losses, due to its higher volatility (see also 3.1.2.2). However, the uncovered gel exhibits less syneresis with increasing ether content. This observation was explained by sealing off pores at the gel/gas boundary caused by fast evaporation (skin formation) due to high volatility of the ether-rich solvent and the non-equilibrium condition of unlimited heads-pace.

Figure 3.3a: Syneresis behaviour of uncovered gels (equivalent to infinite head-space)

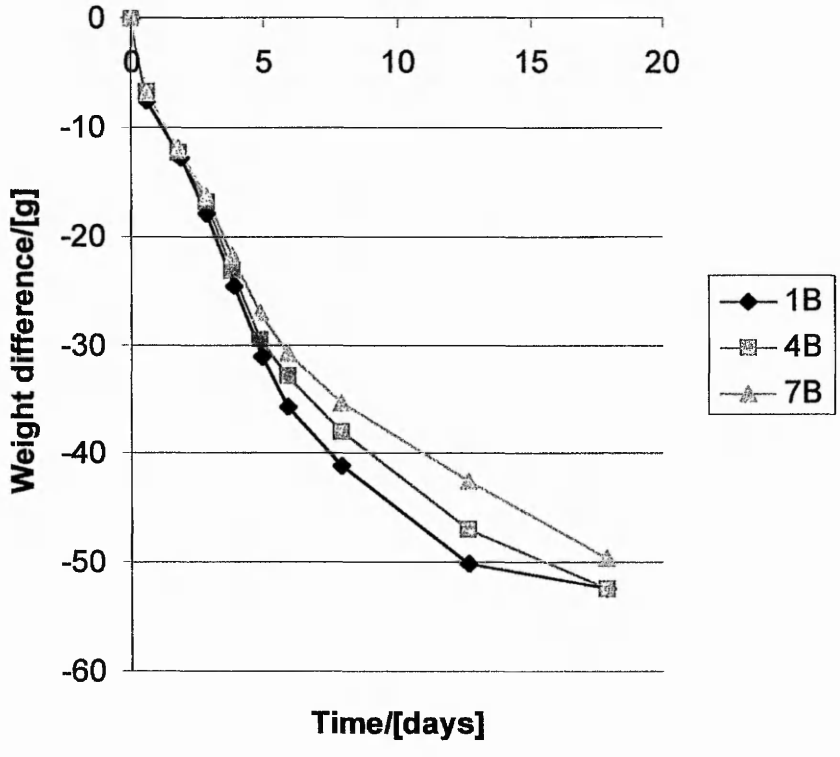


Figure 3.3b: Syneresis behaviour of gels covered with cling film (large – imaginary - head-space)

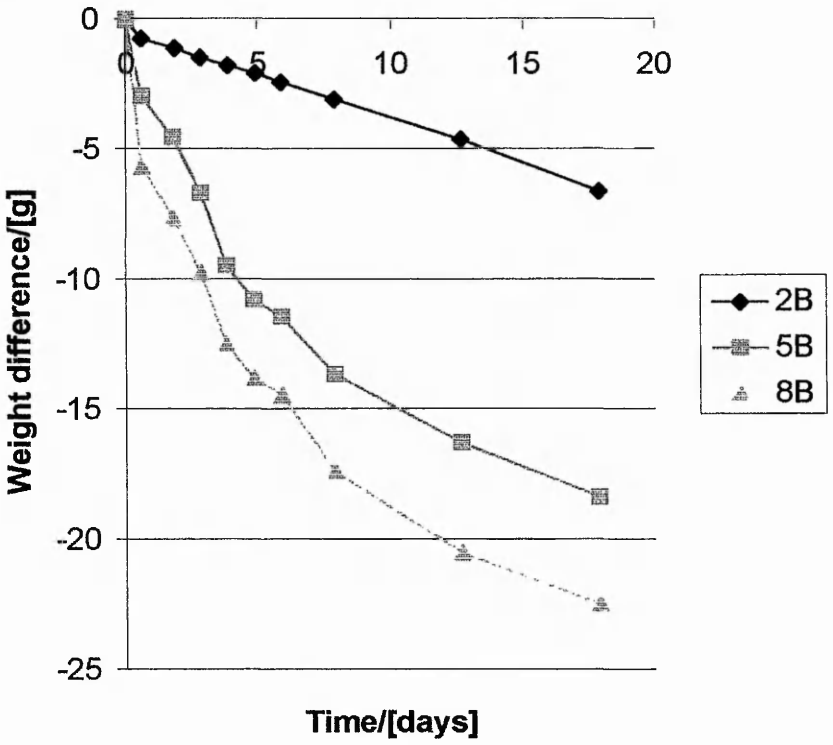


Figure 3.3c: Syneresis behaviour of gels covered with *Parafilm* (small head-space)

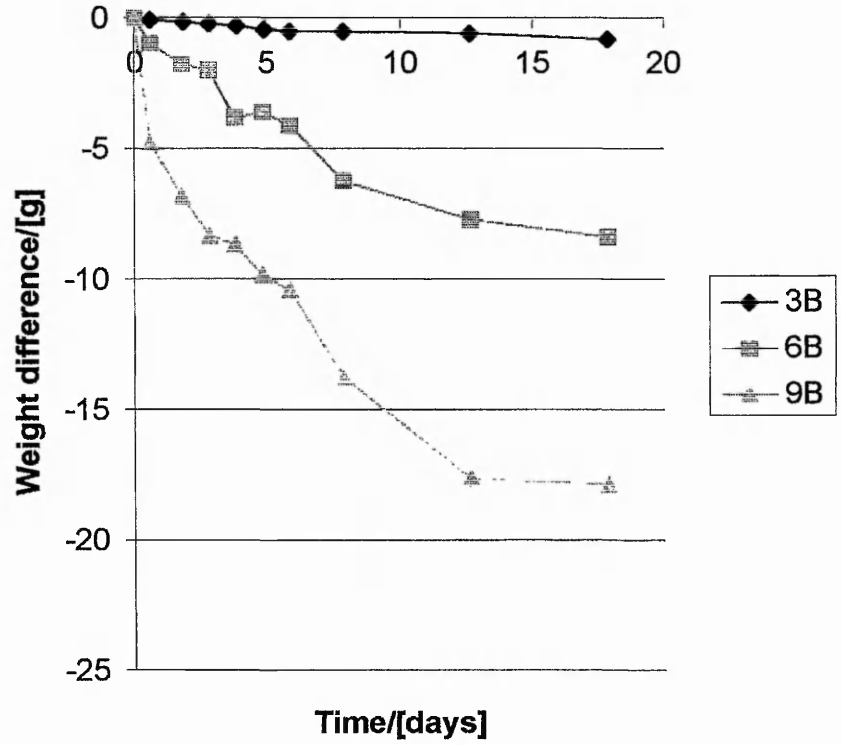


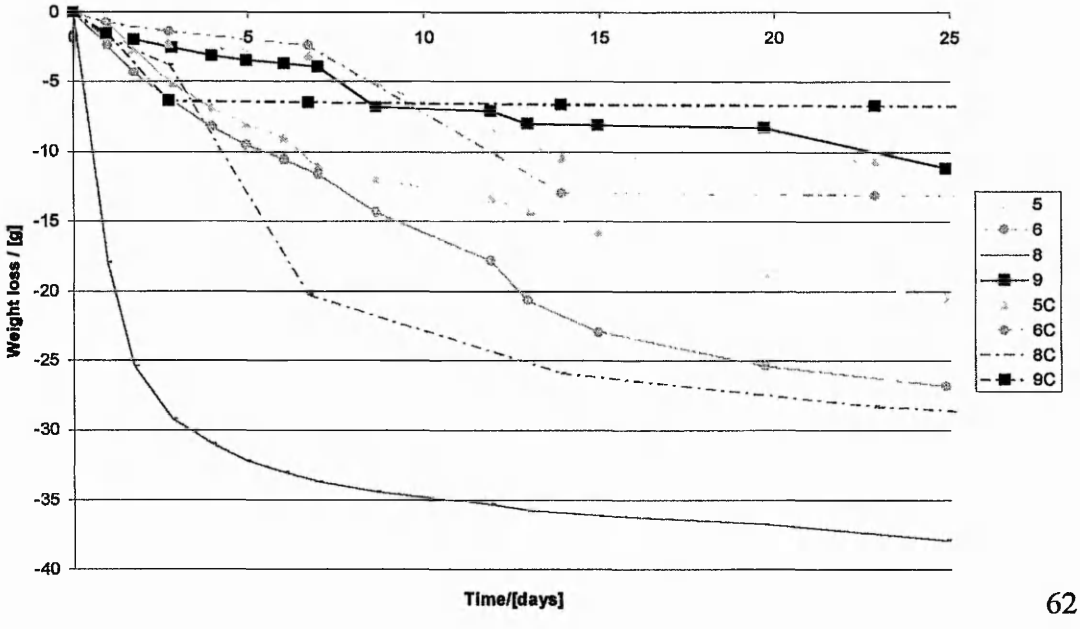
Table 3.3: Composition of reaction systems, reagent amounts and resulting degree of acetylation of series B

Series B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Chitosan solution/[ml]	30	30	30	30	30	30	30	30	30
MeOH/[ml]	26.7	26.7	26.7	17.7	17.7	17.7	13.7	13.7	13.7
Ether/ml	0	0	0	9	9	9	13	13	13
Ac_2O/MeOH 1:10 / [ml]	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Covering	nc	cf	pf	nc	cf	pf	nc	cf	pf
F_A	0.87	0.87	0.88	0.91		0.92	0.91	0.92	0.92

d) Effect of molecular weight

Weight loss due to evaporation in the systems was lower for low molecular weight chitosan (series C), since the number of molecules would be greater and hence the increase of boiling point of the solvent system would be higher than for high molecular weight chitosan (series 0) with fewer molecules per gram. But, as illustrated in Figure 3.4 with examples 5-6, 8-9 and 5C-6C, 8C-9C, syneresis is greater for high molecular weight chitosan due to greater intermolecular forces and lower solubility of higher molecular weight chains, which cause stronger contraction of the gel.

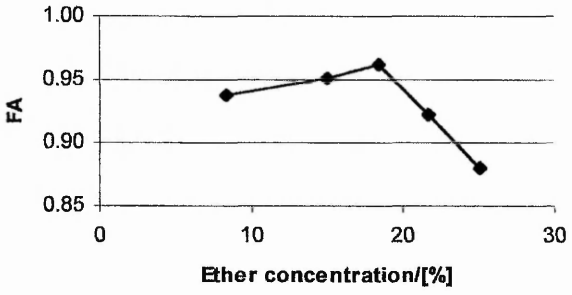
Figure 3.4: Comparison of gels from two different molecular weight chitosans



e) Effect of co-solvent ether on reaction efficiency

It could be shown that there is an optimum ether concentration up to which the reaction of anhydride is getting more efficient and after which the efficiency of the reagent decreases. The optimum ratio of ether/MeOH was 0.85 with an overall ether concentration of 18% at an aqueous/organic ratio of 1:1 and a molar proportion of Ac_2O/-NH_2 of 2.37. The decrease in polarity of the solvent system may work in favour of the therin more soluble higher acetylated material up to a maximum beyond which the decrease of MeOH increasingly counteracts the effect.

Figure 3.5: Degree of *N*-acetylation of series A



3.1.1.3 Summary

Table 3.4: Overview of structure-property relationships in chitosan re-*N*-acetylation systems

Parameter	Effect	Manifestation/consequence
F_A ↑	- Hydrophobicity of the polymer increases, hydrophilicity decreases - Chain stiffness ↑ - Electrostatic charge ↓ → Polymer coil volume ↓	- Polymer solubility in non-polar solvent systems ↑ - Polymer solubility in polar solvents ↓ - Inter and intra polymer chain aggregation ↑ - Syneresis ↑
Ether ↑	- Polarity of solvent system ↓ - Equilibrium shift towards hydrophobic polymer	- Hydrophobic interaction of polymer chain ↓ - Solubility of hydrophobic polymer ↑ - Reaction efficiency ↑ - Onset of gelation shifts to higher values - Clearer gels due to fewer aggregation joints
Anhydride ↑	- F_A ↑ - Polarity of polymer ↓	- Inter and intra polymer chain aggregation ↑ - Syneresis ↑
MW↓	- Solute number/weight ↑ - Boiling point of system ↑	- Evaporation ↓
MW ↑	- Polymer Van der Waals interaction ↑ - Polymer solubility ↓	- Inter and intra polymer chain aggregation ↓ - Syneresis ↑

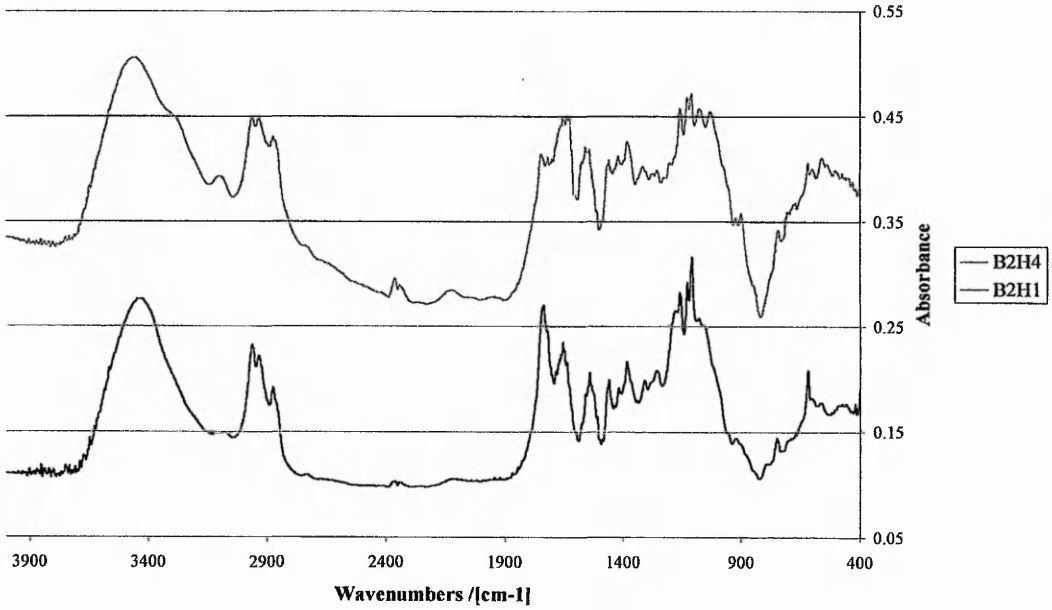
↑ = Increase
↑ = Decrease
→ = Consequently

Relationships between structure of acetyl derivatives of chitosan and their properties in respect to solution - gelation phenomena were observed as above.

3.1.2 Heterogeneous *O*-acylation

Di-*O*-butyrylated chitin soluble in simple organic solvents such as alcohols, acetone, dimethylformamide and methylene chloride has been reported (7, 130). The higher solubility of the chitin derivative is also reflected in markedly lower crystallinity of membranes cast from the material compared to membranes from the initial material chitin (129). The same reaction conditions such as 10 molar excess of butyric anhydride based on hydroxyl groups and 70% perchloric acid as a catalyst were applied to a fine powder of previously *N*-hexanoylated chitosan. The fraction of unreacted amine groups had previously been determined to be [0.02] by dye adsorption. The heterogeneous reaction successfully yielded a Di-*O*-butyrylated-*N*-hexanoylated chitosan, which was soluble in common organic solvents like MeOH, acetone, 1,4-dioxane and slightly in ether. However using butyric anhydride as reagent and as reaction medium would be a rather costly route for preparing commercially feasible membrane materials. Attempts to decrease the amount necessary for reaction by employing

Figure 3.6: Infrared spectra of N-hexanoylated-di-O-butyrylated chitosans



other solvents and by using smaller proportions of reagents were undertaken the idea being that the *N*-hexanoylchitosan can be predicted to be more susceptible to *O*-acylation than chitin, as has been observed for *O*-acetylation (133). Acetone as an alternative reaction

medium was chosen, since butyric anhydride is incompatible with common chemicals such as H_2O and alcohols and HCl as a catalyst, due to the anhydride's incompatibility with strong oxidising agents (218). An equally soluble product could however not be obtained under these conditions, which also made processing and extracting the sometimes highly swollen reaction product from the reaction medium unfeasible. A comparison of the IR spectra of the soluble material B_2H1 and a non-soluble material that could be isolated B_2H4 shows that the ratio of ester to amide absorption bands is 1.70 for the soluble material B_2H1 and 0.81 for the swellable material B_2H4. (Original spectra and peak analysis see Appendix.) This shows that the degree of overall substitution of reactive groups in the chitosan molecule was considerably higher in the easily organo-soluble material than in the non-soluble derivative. Cellulose acetate becomes increasingly hydrophobic and thus soluble in organic non-polar solvents in proportion to its overall degree of substitution. (DS up to 3.0)(131) By analogy, *N*-acyl chitosan should become more organo-soluble with increase in the extent of *O*-substitution and hence decrease in hydrogen bonding present.

3.1.3 Homogeneous *N*-carbamylation

3.1.3.1 Introduction

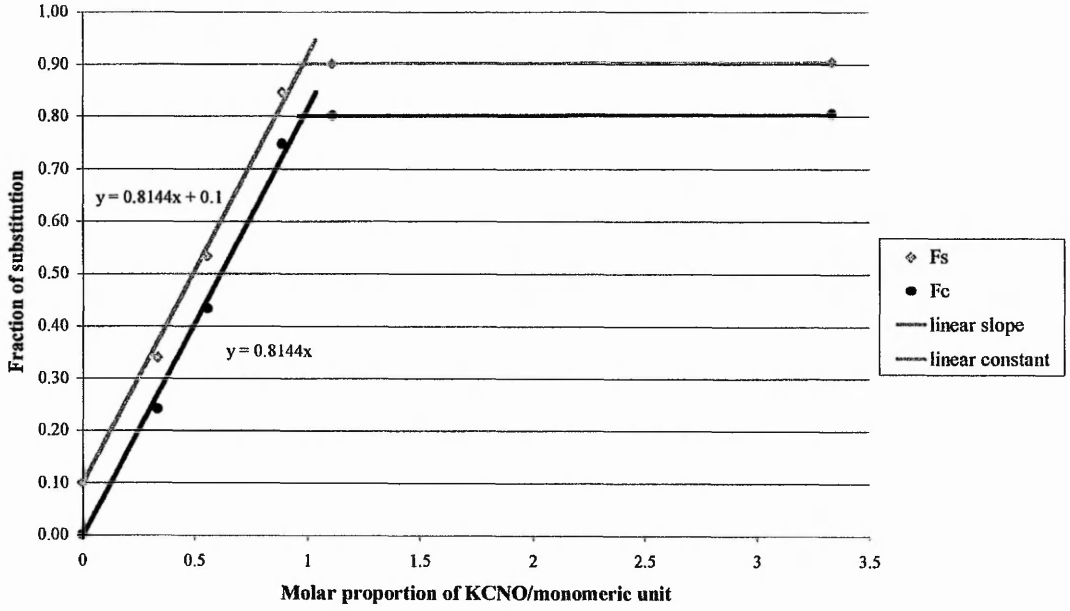
Chitosan can be carbamylated to R-NH-CO-NH_2 with potassium isocyanate in aqueous and aqueous/methanolic medium (219),(220) forming turbid white not very elastic gels which show quick fungus growth at low acid concentrations and in the absence of MeOH. Since KCNO decomposes in acid solutions to CO_2 and NH_3 (221) the HOAc concentration needs to be kept to the minimum required for the dissolution of chitosan. MeOH was not required for the reaction, but was added for comparison of *N*-carbamyl gels with *N*-acyl gels.

3.1.3.2 Reaction efficiency

Aqueous solutions with chitosan and HOAc concentrations of 1% and 0.05M respectively were reacted with a range of molar proportions of KCNO to amine group. Figure 3.7 shows that the degree of reaction increases linearly with a proportion of 81% substituted amine groups per initial number of KCNO up to $F_C \approx [0.7]$ (black graph). After this linear region the gradient of substitution over amount of reagent quickly decreases to zero and

after F_C=[0.8] and F_S=[0.9] at a molar proportion of 1.1 no further substitution could be obtained by increasing the amount of KCNO to 3.3. Under homogeneous conditions substitution would be expected to progress randomly along the chain and hence carbamylation beyond F_C=[0.8] and F_S=[0.9] could be hindered by steric effects.

Figure 3.7: Relationship between molar proportion of KCNO and degree of substitution



The grey graph shows the overall substitution of the chitosans starting with an initial F_A=[0.10]. The analysis of substitution was carried out by dye adsorption (3). The linearity of reaction efficiency also shows that the carbamyl group is not protonated in 0.1M HOAc.

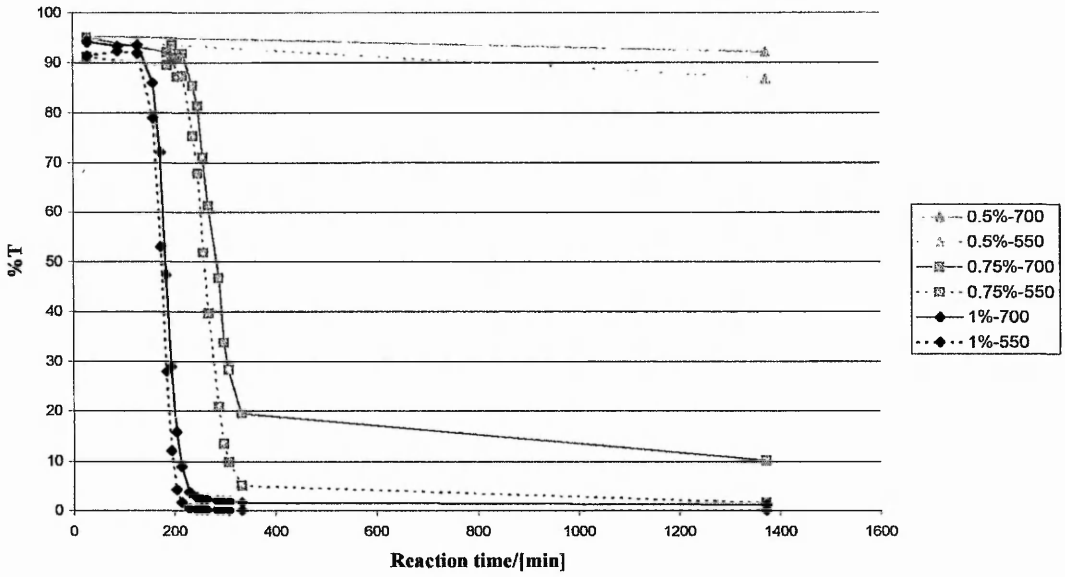
3.1.3.3 Influence of polymer concentration, degree of substitution and molecular weight on gelation

At a molar proportion KCNO/-NH_2 of 1:1 gelation was observed at polymer concentrations ≥ 0.5%, which compares well to reported values for chitosan *N*-acyl gels (213).
Gelation started at F_S=[0.5] and F_C=[0.4] which is lower than values for reported *N*-hexanoyl chitosan gels which exhibit gelation from F_H≈[0.7](213).
Lower molecular weight chitosan did not form a turbid white gel, but precipitated in the process of reaction to F_C>[0.5] as white flocs.

3.1.3.4. Turbidity and its implications for the gel structure

The turbidity of the gels was monitored over reaction time by transmission measurements at 700nm and 550nm against 1% chitosan solution as a reference.

Figure 3.8: Transmittance of *N*-carbamyl chitosan gels with different polymer concentrations over reaction time

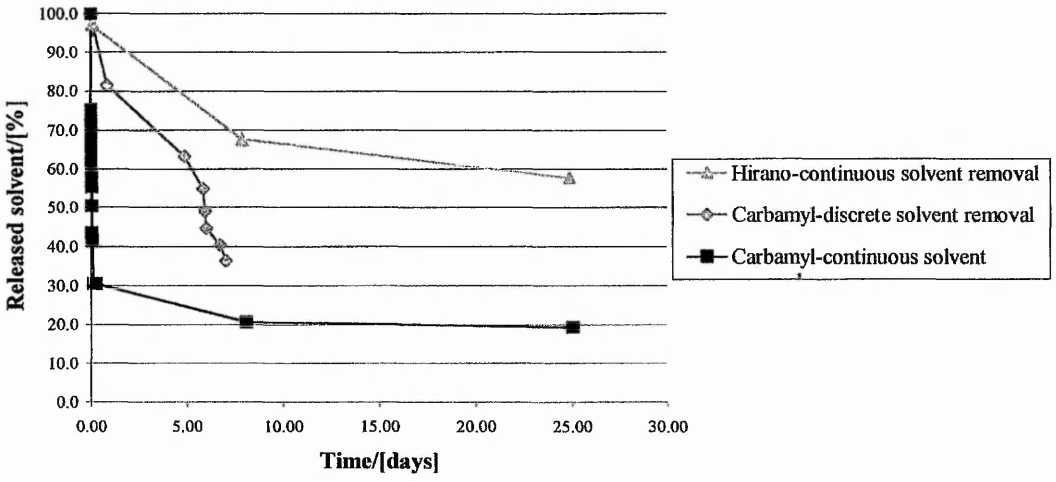


Three reaction systems were prepared at a molar proportion of 1:1 KCNO/-NH_2 at polymer concentrations between 0.5% and 1%. The gel with a concentration of 0.5% did not show visible clouding, which is reflected in a nearly constant high transmittance in Figure 3.8. The higher concentration gels exhibited increasing opacity over time and their transmittance dropped by over 70% within some 6h of reaction. Testing the gels for crystalline regions by observation under polarised light was negative and did not lead to the conclusion that the turbidity was caused by crystalline alignment of the chains at the junction points. As described in 3.1.3.3 the polymer exhibited precipitation at lower molecular weight and higher substitution. Hence the turbidity could be caused by partial precipitation of the polymer to amorphous flocs within a gelatinous network at certain polymer concentrations and molecular weights.

3.1.3.5. Syneresis behaviour

The syneresis in carbamyl gels was strongly dependent on the process of solvent removal. Syneresis was lower in undisturbed gels, but showed a drastic increase when the gel was touched during the monitoring process, or when solvent was removed at shorter time intervals. In Figure 3.9 the syneresis behaviour two carbamyl gels, one with discontinuous and one with continuous solvent removal were compared to a Hirano (homogeneously acylated chitosan) gel. Continuous solvent removal was achieved by continuous drainage of the gel with the solvent being collected in a measuring cylinder under sealed conditions.

Figure 3.9: Syneresis of carbamyl gels with different solvent removal compared with a Hirano gel



Hirano gels do not show the same behaviour as carbamyl gels. Their solvent release curve can be approximated by a function of the order $x^{-1}+c$ (hyperbolic) independent of solvent removal intervals. (222) The differences in syneresis behaviour indicate that carbamyl gels have got a different structure to Hirano gels. The latter have been reported as polyphasic with solvent filled pores of diameters <50μm and lengths <300μm. (223) The present results suggest that besides a polymer network with junction points and pores there are precipitation areas, which lead to turbidity and mechanical weakening of the gel. The fact that undisturbed and thus intact carbamyl gels are holding solvent very well can be explained by phase border phenomena. As the reaction progresses gelation sets in first and clouding develops later (see 3.3.4). This means that as hydrophobicity increases after the onset of gelation the solubility of the polymer decreases up to a point where partial precipitation within the gel network occurs. The observation that the gel does not

disintegrate in this process means that on the phase border between head-space and vessel wall the chains do not precipitate but exist in the gel state, while precipitation only occurs within the gel network in contact with the more polar solvent.

3.1.3.6 Solubility of N-carbamyl chitosan

N-carbamyl chitosan formed clear solutions in 0.1M HOAc up to a mole fraction of substitution of [0.62] and dissolved to cloudy solutions/dispersions up to F_S [0.75]. This behaviour is not dissimilar to re-*N*-acetylated chitosans which are soluble in acetic acid up to $F_A \geq$[0.70] (224) (see also section 3.3). The similarity is not surprising, since the substituent only varies in its $-NH_2$ group instead of the $-CH_3$ group. The former group is not a very eager proton acceptor, which can be seen in the course of dye adsorption. This could be a consequence of keto-enol tautomerism, which attracts the free nitrogen electron pair towards the neighbouring C-atom.

3.1.4 Crosslinking

3.1.4.1 Introduction

Crosslinking in chitosan has got two functions. It prevents dissolution of the polymer in aqueous media at low pH values and can be used in order to control pore sizes of membranes. (72, 122, 124, 127) Probably the most commonly used crosslinking reagent in connection with chitosan is glutaraldehyde (225). However glutaraldehyde crosslinked materials would not be acceptable in the treatment of fluids for later human or animal ingestion. Thus multifunctional acids such as citric acid and itaconic acid were investigated for their potential as crosslinking agents for chitosan.

3.1.4.2 Crosslinking with itaconic acid and citric acid

Chitosan was successfully crosslinked with itaconic $H_2C{=}C(COOH)\text{-}CH_2\text{-}COOH$ (2 reactive sites) and citric acid $HO\text{-}C(COOH)\text{-}(CH_2\text{-}COOH)_2$ (3 reactive sites) to yield films that were insoluble in dilute 0.1M HOAc. Chitosan was reacted with citric acid in molar proportions ranging from 0.5 to 3 and with itaconic acid in a range of 1.6-6.5 based on free amine groups. For this the chitosan was dissolved in 0.2M HOAc, made up to a 1% polymer solution with the respective reagent solution. After drying the films were cured under defined conditions. The temperature ranged from RT (approximately 21°C) up to

100°C. The effect of crosslinking on the integrity of films in agitated 0.1M HOAc can be seen in Table 3.5 for citric acid and Table 3.6 for itaconic acid.

Table 3.5: Solubility of chitosan films crosslinked with citric acid in 0.1M HOAc

Curing time: 1.25h	**Molar reagent/chitosan proportions based on free amine groups**			
Curing temperature	0.5	1.0	1.5	2
RT	+	+	+	+
40°C	+	+	+	+
70°C	+	+	+	+
100°C	-	-	-	-

\+ = Dissolution

\- = Swelling

Table 3.6: Solubility of chitosan films crosslinked with itaconic acid in 0.1M HOAc

Curing time: 1.25h	**Molar reagent/chitosan proportions based on free amine groups**			
Curing temperature	1.6	2.4	4.8	6.5
RT	+	(+)	(+)	(+)
40°C	+	(+)	(+)	(+)
70°C	+	(+)	(+)	(+)
100°C	+	-	-	-

\+ = Dissolution

(+) = Partial dissolution with disintegration of film to flakes

\- = Swelling

It can be seen that citric acid is a more suitable crosslinking agent than itaconic acid. Citric acid treated films are more resistant to acid medium even at lower reagent proportions. This is a result of more crosslinks formed per mole of reagent added, as would be expected because of the greater functionality of citric acid. A measure for the amount of crosslinks formed is the EW of amine groups in the network. In Table 3.7 it can be seen that the EW

of citric acid reacted materials is much higher compared to the ones treated with itaconic acid than can be accounted for by lower formula weight of itaconic acid compared to citric acid. The fraction of free amine, as well as the absolute number of crosslinks can not be easily determined by this method. This is due to the fact that not every reactive site of the crosslinking agent may have reacted, but there may be residual reactive end groups from the reagent, which can not be quantified by dye adsorption with Acid Orange 7.

Table 3.7: Assessment of chitosan crosslinking by dye adsorption

Crosslinking agent	Citric acid	Citric acid	Itaconic acid	Itaconic acid
Molar proportion	1.5	2.0	4.8	6.5
EW/[g/mol]	429	438	315	334
Idealised m_0/[g/mol]	207	207	192	192

Citric acid treated films show hydrophilicity even at ambient humidity giving them unexpected adhesive properties, which might be useful for wound treatment. The reaction of both reagents is temperature dependent and clearly shows that a temperature of 100°C is necessary in order to obtain sufficient reaction to achieve insolubility in aqueous media. This can be easily explained by the fact that the reaction

$P\text{-}NH_3^+ \ ^-OOC\text{-}P \rightarrow PNH\text{-}CO\text{-}P + H_2O$

(P = polymer)

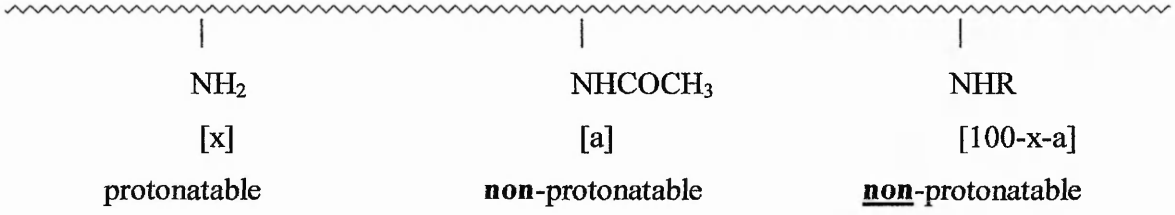
is speeded up with increase in temperature, or has an activation energy that requires temperatures of around 100°C.

3.1.5 Derivation of relationships for EW and *N*-substitution for non-protonatable secondary substituents differing from acetyl groups

Deriving from the principle applied for chitin and chitosan (3) (see Introduction 1.5.2.4) a formula for the facile and inexpensive determination of the degree of secondary substitution for a non-protonatable secondary substituent can be developed. The varying contributions which substituents with a size different from that of the acetyl group make to

the weight of the material and the EW respectively have been considered. The formula is not specific to *N*-acyl derivatives, but can be employed for any other non-protonatable group with $S = m_0$ of the respective substituted sugar unit. The initial F_A is required to be determined before further substitution of the material.

Figure 3.10: Schematic substitution conditions along a derivatised chitin and chitosan chain respectively with only amine as protonatable group



$$EW = \frac{x*161 + a*203 + (100 - a - x)*S}{x}$$

(not defined for x = 0, that is in the absence of protonable groups)

Above formula is derived from the definition of EW, namely the weight of monomeric units over the amount of protonatable groups, equating to x, since only the amine group is basic in the polymer model regarded.

$$\Rightarrow x = \frac{(203 - S)*a + 100*S}{EW - 161 + S}$$

$$D_S = 100 - x$$

$$F_S = \frac{Ds}{100} = 1 - \frac{x}{100}$$

$$F_N = Fs - Fa$$

EW = equivalent weight of amine group

x = percentage of anhydro-D-glucosamine units

a = percentage of anhydro-*N*-acetyl-D-glucosamine units

S = monomer molecular weight of anhydro-D-glucosamine units *N*-substituted with R

D_S = overall degree of substituted sugar units

F_S = overall fraction of substituted sugar units

F_N = fraction of secondary substituted sugar units

For detailed derivation and examples see Appendix.

3.1.6 Overview of sources for troubleshooting in F_A analysis by dye interaction

Various experimental as well as systematic errors can lead to false results in the determination of the EW and thus F_A or F_S by dye analysis. In Table 3.8 a comprehensive overview is given of phenomena that affect different dye interaction methods, their consequences and ultimately their effect on the obtained value for EW. Making due allowance for the factors below ensures dye adsorption to be a reliable method for the analysis of substitution degrees.

Table 3.8: Checklist for possible sources of inaccuracies for substitution analysis of chitosan by dye interaction

Phenomenon	**Affected methods**	**Consequence**	
Dissolution of low molecular weight fractions or higher soluble chitosan derivatives	Dye uptake	- Metachromatic shift, λmax moves, and measurement is taken at lower absorbance values (leads to seemingly higher dye removal, higher –NH_2/protonatable groups concentration, lower EW) - Less adsorptive material (leads to less dye removal, seemingly lower –NH_2/protonatable groups concentration, higher EW)	Active sites ↑ EW↓
		Both effects counteract each other, but it is not clarified if they level each other out	Active sites ↓ EW↑
Low MW fractions (e.g. prior degradation) not taking part in interaction	Metachromatic titration	Less amines interact and the EW is seemingly higher and the –NH2/protonatable groups concentration seemingly lower.	Active sites ↓ EW↑

Phenomenon	**Affected methods**	**Consequence**	
Water adsorbed on not completely dried material, other contaminations	Dye uptake, metachromatic titration	Amine concentration is lower than in completely dry material, thus EW seems higher and $-NH_2$/protonatable groups seems lower.	Active sites ↓ EW↑
Material not completely dissolved	Metachromatic titration	Not all $-NH_2$/protonatable groups are taking part in the metachromatic interaction, the EW is seemingly higher and the fraction $-NH_2$/protonatable groups appears lower.	Active sites ↓ EW↑
Solvent loss through evaporation	Dye uptake	The dye solution is more concentrated this results in seemingly lower dye removal, the $-NH_2$/protonatable groups concentrations appears lower and the EW higher.	Active sites ↓ EW↑
Polymer/dye residue in filtrate	Dye uptake	Diffuse adsorption at T < 60°C when the filtrate is cooling down leads to additional unstoichiometric adsorption onto the polymer. The EW appears lower and the amino group concentration higher.	Active sites ↑ EW↓
Material not completely accessible for dye interaction	Dye uptake of especially chitin and highly hydrophobic chitosan derivatives	Dye molecules do not occupy all active sites.	Active sites↓ EW↑

3.1.7 Sodium *N*-methylsulphonation of chitosan with FSBS

The implications of sodium *N*-methylsulphonation of chitosan with FSBS are elucidated in detail in sections 3.2 and 3.3.

3.1.8 Summary

- Relationships between structure of chitosan/chitin intermediates varying in F_A and molecular weight and their behaviour in gelation systems with differing solvent compositions were established (details see Table 3.4).
- The reaction efficiency of homogenous *N*-acetylation of chitosan in aqueous MeOH systems was improved by the addition of ether as a co-solvent.
- Successful manipulation of hydrophilicity of chitosan by introduction of acyl substituents was achieved
- A novel chitosan film and fibre forming derivative *N*-hexanoyl-di-O-butyryl-chitosan soluble in common organic solvents like MeOH, acetone, 1,4-dioxane and slightly soluble in ether was prepared successfully.
- A non-published reaction namely carbamylation of chitosan with potassium isocyanate was investigated.
- The reaction efficiency of chitosan carbamylation was determined to be linear with a factor of 0.8, corresponding to approximately 100% efficiency (KCNO purity 80%) up to an overall fraction of substitution of [0.9], after which the fraction of substitution over the molar proportion of reagent added was constant.
- The structure of the gels occurring in the course of carbamylation was determined to be different to standard Hirano gel. While the latter are rigid and polyphasic with a homogeneous appearance, the former are only homogeneous networks at low concentrations, but form networks with included precipitation regions that are, however, non-crystalline according to polarised light.
- The solubility of the derivatives was observed to be similar to re-*N*-acetylated chitosan with clear solutions forming up to F_S [0.62] and cloudy solutions forming up to F_S [0.75]
- Chitosan films were successfully crosslinked with citric acid and itaconic acid to yield acid resistant membranes.
- EW- F_A relationships established for chitin and chitosan (3) were mathematically modified to yield valid formulae for the determination of the fraction of substitution by non-protonatable secondary subsituents (residual or introduced acetyl groups being considered as primary substituents) from experimentally determined EW.

3.2 Preparation and characterisation of sodium *N*-methylsulphonated chitosan (CHMS)

3.2.1 Preparation and isolation of CHMS (see Figure 3.36b)

3.2.1.1 Dissolution reprecipitation process

Solid chitosan can be reacted with FSBS to CHMS in neutral (pH range 5 – 9) aqueous medium in a patented process (142). The reaction can be seen as dissolution of chitosan with progressing complex formation. The sodium *N*-methyl sulphonate (-CH_2-SO_3Na) groups introduced are strongly dissociated as indicated by the fact that sulphonic acids reach and exceed the solubility of some mineral acids (226). The complex was isolated from the solution formed by reprecipitation in organic non-polar solvents that are inert to FSBS such as acetone. For further processing see section 3.2.1.4. Material produced by precipitation dissolves at a faster rate in H_2O than heterogeneously reacted polymer (see 3.2.1.2). Similar solubility behaviour has been observed for highly substituted chitosan when recovered by precipitation in organic solvent (209). The yields in Table 3.9 are very narrowly distributed around 85%, determined with consideration of different m_0 values, and do not show a trend for different DPD and F_A. This strongly indicates that the DPD remains constant during reprecipitation.

Table 3.9: Yields of CHMS prepared by dissolution-reprecipitation from three different chitosans varying in their molecular weight and degree of primary *N*-acetylation

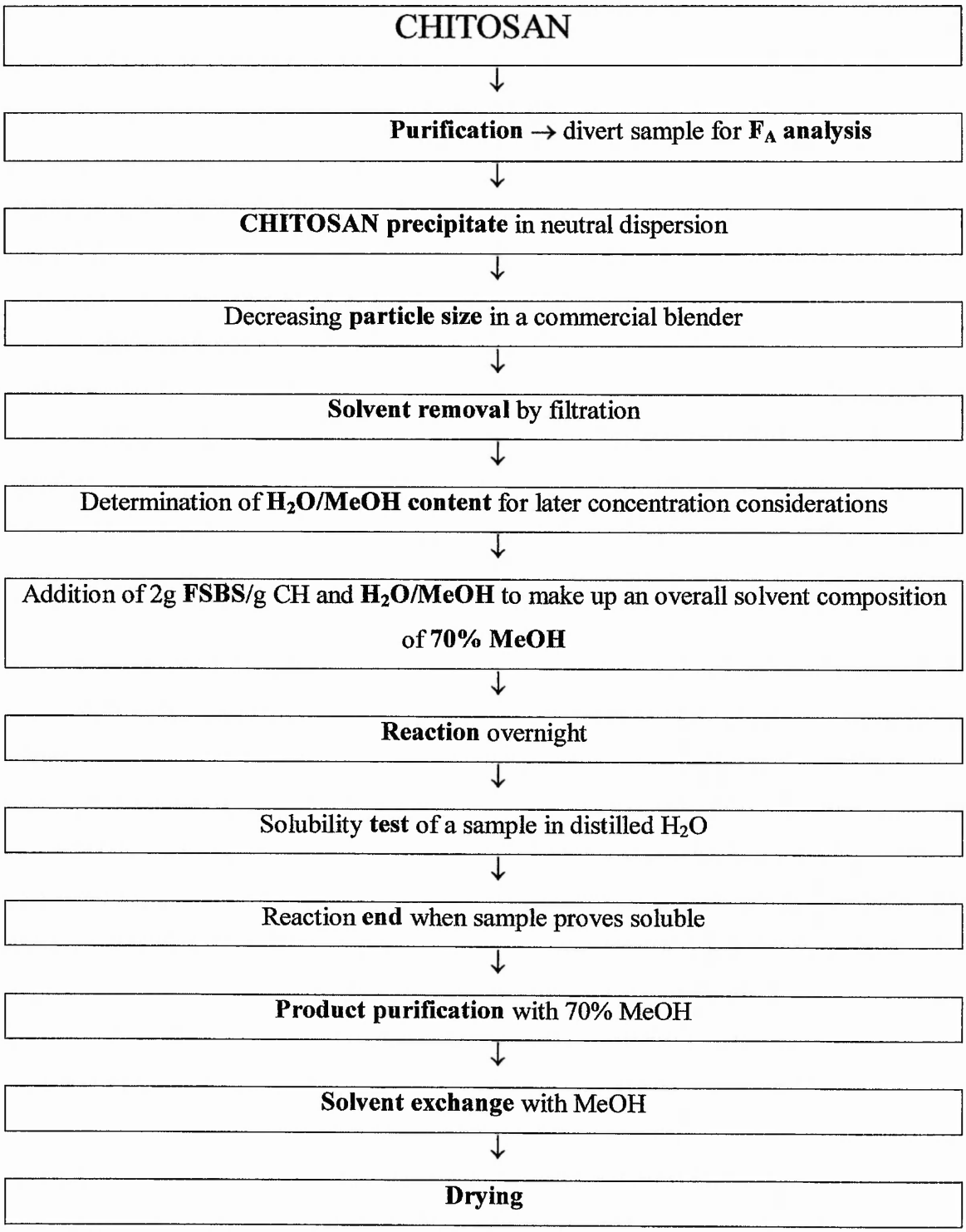
CHMS sample	yield/[%]
CHMS-H[0.11]	85.01
CHMS-M[0.11]	86.24
CHMS-L[0.25]	84.04

H: high MW, M: medium MW, L: low MW

3.2.1.2 CHMS prepared from never-dried chitosan

CHMS samples NCm1 and NCm2 were prepared by heterogeneous reaction of never-dried chitosan in methanolic FSBS solutions. Heterogeneous reactions have got several

Figure 3.11: Flow-plan for the preparation of CHMS from chitosan via never-dried material



advantages. Higher polymer amounts are reacted in smaller volumes and additionally the drying step after purification becomes redundant, which is cost and time effective as well

as being a gentler process. A third advantage is a more facile recovery eliminating an otherwise essential but solvent-intensive reprecipitation step. Never-dried chitosan was produced in the course of standard purification.

From the purified material a sample is removed for drying and subsequent F_A analysis. The latter needs to be known for later determination of secondary substitution. The material is filtered to remove the main bulk of solvent. This is important so that MeOH and subsequently FSBS dissolved in H_2O can be added to make up a dispersion with a solvent composition of 70% MeOH v/v to allow reaction while at the same time preventing the polymer from dissolving. An overview of the process steps is given in Figure 3.11. Metachromatic titration confirmed the material as fully *N*-substituted.

3.2.1.3 General processing requirements

For purification and removal of unreacted FSBS from CHMS precipitates or particles in general the material was washed with aqueous solutions of MeOH, a solvent for both FSBS and side products. The proportions of MeOH to H_2O depend on whether a priority is the retention of all fractions and especially low molecular weight material, or an optimisation of overall material yield over solvent expenditure. For ensuring retention of the overall molecular weight composition as well as maximising the absolute yield a MeOH concentration of 75% has been employed. With decreasing FSBS concentration in the overall solvent system the material is in contact with, the hydrophilicity and solubility of the material increase. Strong swelling of CHMS particles washed in aqueous MeOH and spread out on a tray to ensure efficient drying in an aerated drying cabinet has been observed. The swelling and subsequent agglomeration lead to an increase in particle size and decrease in surface area. The reason is a depletion of MeOH in the contact solvent and consequently an increase in H_2O concentration so that the material swells strongly. Where particle size plays a crucial role for further application of the material the swelling can be avoided by solvent exchange of residual H_2O with e.g. MeOH after quantitative purification but prior to drying.

3.2.1.4 Stability of CHMS in solution

CHMS can be dissolved to form solutions of up to 15% by weight (142). These highly concentrated solutions in H_2O have been found stable for periods of time >15 months at RT under sealed conditions (142). On longer standing such a solution has been found to discolour and gelate (219).

More dilute aqueous solutions of 0.05% to 2% were found to show varying degrees of stability. Clouding was found to set in within days especially for very dilute solutions. This was seen as an indicator of reversion of the CHMS towards the initial chitosan material by disintegration of the complex due to a shift in the equilibrium

$$\text{Chit-NH}_2 + \text{HOCH}_2\text{SO}_3\text{Na} \rightleftharpoons \text{Chit-NHCH}_2\text{SO}_3\text{Na} + \text{H}_2\text{O}$$

towards the left hand side due to low FSBS as well as polymer concentration, while in higher concentration the polymer acts as its own hydrocolloid.
The metastability of CHMS was considered in determination of dilute solution viscosity, where the standing time was kept to the minimum required for dissolution and measurement. A solution of 2% concentration exhibited clouding after weeks on standing and eventually precipitation of the material to a coherent sludge in the off white colour of typical chitosan particles.

3.2.2 Gravimetric determination

Acid hydrolysis and subsequent gravimetric analysis was used to determine the degree of reaction in CHMS. The principle is heterogeneous and quantitative scission of the methylsulphonic acid group from the chitosan backbone and weight assessment before and after acid hydrolysis. The results take into account, that the initial chitosan was identified as a material with a fraction of *N*-acetylation of [0.156] and thus m_0 of the initial material was 199g mol^{-1} rather than 161g mol^{-1} as for fully de-*N*-acetylated chitosan. In a strong mineral acid the acidic substituents are protonated rendering the polymer insoluble in the medium. Acid hydrolysis in H_2SO_4 cleaves the side groups recovering chitosan, which is reported insoluble in dilute H_2SO_4 (154).
The first series was carried out in small beakers and the precipitate transferred to glass crucibles for filtration and washing.

Table 3.10: Reaction parameters of initial gravimetric tests of CHMS

CHMS sample	S1	S2	S3	N8
Chitosan source process	Never dried, Heterogeneous reaction	Ball milled, Heterogeneous reaction	Hammer milled, Heterogeneous reaction	Reprecipitated from solution in acetone
Morphology	Crude particles	Very fine powder	Fine sugar like	Electrostatic flakes (very fine particles)
Reaction medium	2N H_2SO_4	2N H_2SO_4	2N H_2SO_4	2N H_2SO_4
Swelling on addition of above solvent (1=most)	2	4	1	3
Partial dissolution	+	+	+	+
Complete dissolution before reprecipitation	After approx. 7h	Not observed	Not observed	After approx. 8h
Reaction time	Approx. 24h	Approx. 24h	Approx. 24h	Approx. 24h
Washing medium 1	Methanolic ammonia	Methanolic ammonia	Methanolic ammonia	Methanolic ammonia
Washing medium 2	50% MeOH	50% MeOH	50% MeOH	H_2O dest
Washing medium 3 (after drying, over-night steeping)	Methanolic ammonia	Methanolic ammonia	Methanolic ammonia	-
Swelling on addition of distilled H_2O (1=most)	3	1	2	-

+ = affirmative

The fact that some of the compounds were observed to dissolve at some point of the hydrolysis before reprecipitation occurred indicates that there is an intermediate degree of substitution, at which the compound becomes soluble in the reaction system (2N H_2SO_4). N8, after being washed with methanolic ammonia, swelled strongly on washing with H_2O. This indicated that the hydrolysis reaction had not been complete. The N8 was discarded. However the dried samples S1-S3 were immersed in methanolic ammonia under agitation

over-night in order to remove acid if present and subsequently test for swellability. The samples S1-S3 had been washed with 50% MeOH after neutralisation with methanolic ammonia. A subsequent test for swelling in H_2O was positive.

After being steeped in methanolic ammonia the very brittle samples were steeped in H_2O over night and all of them showed swelling. While S2 almost completely disintegrated S1 and S3 still kept their morphology and exhibited only slight swelling, especially S1. Since a quantitative removal of the substituent group had not been achieved a second series was devised changing the following parameters. Since particle size is crucial in heterogeneous reactions in general, sample N8 was chosen for further hydrolysis since it had the largest surface area per gram weight.

This time the hydrolysis itself was carried out directly in scintered glass crucibles in order to eliminate a transfer step and so minimise possible material loss. The crucibles were placed into small beakers containing H_2SO_4 in different reaction systems with varying polarity (see Table 3.11) to avoid swelling and agglomeration, leading to a decrease of surface area, and also to have a good solvent for the leaving molecule (organic phase) and 2N H_2SO_4. The dispersions were agitated with magnetic stirrers. It was important to agitate the mixtures immediately, after addition of solvent, as slight swelling occurred even in 75% methanolic solution, and

Table 3.11: Reaction parameters of second modified gravimetric test series of CHMS

CHMS sample	**N8 (A)**	**N8 (B)**	**N8 (C)**	**N8 (D)**
Reaction medium	2N H_2SO_4 75% MeOH	2N H_2SO_4 50% MeOH	2N H_2SO_4 50% EtOH	2N H_2SO_4 75% MeOH
Swelling on addition of solvent (1=most)	3	1	2	3
Partial dissolution	-	-	-	-
Reaction time/[h]	64	64	64	64
Washing medium 1	Methanolic ammonia	Methanolic ammonia	Methanolic ammonia	Methanolic ammonia
Washing medium 2	75% MeOH	50% MeOH	50% EtOH	75% MeOH
Determined F_S	1.04	0.87	1.07	0.59

- = none observed

the powder otherwise formed a disk similar to foam rubber. The latter leads to a drastic decrease of surface area in contact with the reaction medium. The reaction time was increased to ensure complete reaction.

The sample N8 (D) shows with F_S =[0.59] a seemingly low value of substitution, attributed to the fact that the powder formed a swollen disk on addition of the solvent/reagent system and thus hydrolysis as well as removal of cleavage product were not complete. N 8 (B) has with 0.87 a low value compared to N8 (A) and N8 (C) with 1.04 and 1.07 respectively, which is not outside the accuracy band of this gravimetric method. Theoretically the F_S can not exceed a theoretical value of 1.00. However due to involuntary substance loss during the several steps of the gravimetric determination determination the values above 1.00 arise from experimental error and are taken to indicate complete secondary substitution of the initial material.

For optimisation of the gravimetric determination it was repeated in 50% ethanol as solvent system, since the solvent system yielded good results in the second series and EtOH is the better solvent for the cleaved product. The latter has got two advantages, namely the easier removal of by-product and even more important shifting the equilibrium favourably. The reaction time was chosen at 16 h, since this would present a feasible time scale for practical purposes. Three (3) samples were determined under the same conditions to obtain a measure for the reproducibility of the method.

Table 3.12: Reaction parameters of optimised gravimetric tests of CHMS

Sample	**N8 (E)**	**N8 (F)**	**N8 (G)**
Reaction medium	2N H_2SO_4 50% EtOH	2N H_2SO_4 50% EtOH	2N H_2SO_4 50% EtOH
Reaction time/[h]	16	16	20
Washing medium 1	discarded due to accident	Methanolic ammonia	Methanolic ammonia
Washing medium 2		50% EtOH	50% EtOH
determined F_S	-	1.08	1.19

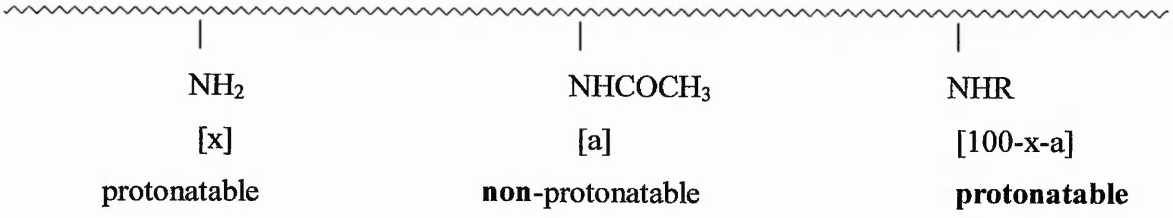
The method contains inaccuracies in itself, due to swelling behaviour and diffusion issues, which are obstructive to complete hydrolysis. The possibility of incomplete hydrolysis, which would give rise to seemingly lower values of substitution and the potential weight

difference due to material loss which would yield seemingly higher values have to be considered. Thus the results of hydrolysed values over residual monomeric units show a comparatively high spread. The results obtained for N8 (F) and N8 (G) compare very well with those obtained for N8 (A) and N8 (C). All of these values are above the theoretically possible value of 1.00, which indicates quantitative hydrolysis with negligible overall material loss during the process. It can be concluded that the water soluble CHMS N8 produced by dissolution/reprecipitation was fully substituted.

3.2.3 Derivation of relationships for EW and *N*-substitution for protonatable secondary substituents differing from amine groups

Degrees of protonatable *N*-substitution can be determined by a facile and inexpensive dye interaction method similarly to that described in section 3.1.5 for non-protonatable secondary substitution.

Figure 3.12: Schematic substitution conditions along a derivatised chitin and chitosan chain respectively with only amine and secondary substituent as protonatable groups



When the EW is determined experimentally for a specific sample, rather than calculated for theoretical curves, then the fraction of all protonatable amine groups will be determined, as these will be the interacting species in metachromatic and dye adsorption processes. This explains why in the following formula the denominator consists of the percentage of both substituents and the numerator is the sum of the products of the m_0 and the respective percentage in which they occur along the polymer chain. Furthermore [x] occurs in the numerator, as this determines the contribution of glucosamine groups to the molecular weight of the polymer, while it does not appear in the denominator, as the latter consists of all protonatable groups, which also includes the [100-a-x] portion which is the percentage of secondary substituents. In the final result, when an experimentally determined EW is employed to solve the equation and determine the degree of substitution,

[x] is the percentage of all protonatable amine groups, in this case the sum of $-NH_2$ and –NHS.

$$EW = \frac{x*161 + a*203 + (100-a-x)*S}{100-a}$$

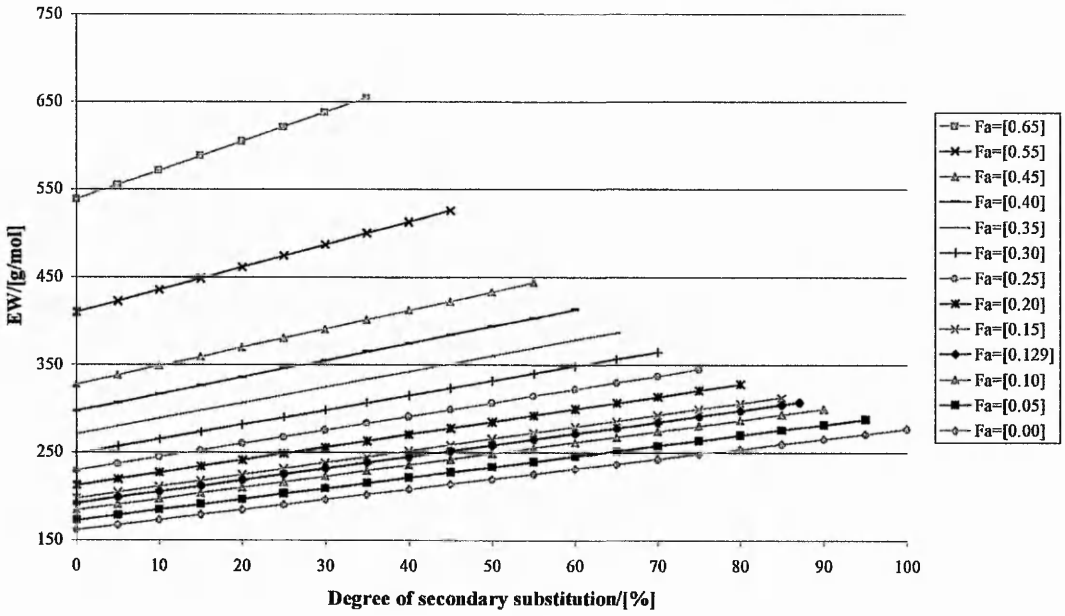
$$x = \frac{EW*(100-a) - (203-S)*a - 100*S}{(161-S)}$$

S = m_0 of fully R substituted anhydro-D-glucosamine unit

x = percentage of anhydro-D-glucosamine units

For a detailed derivation see Appendix.

Figure 3.13: Equivalent weights of the amine group in CHMS-chitosans with different F_A and at varying degrees of secondary substitution



The equation initially includes two unknown variables in the percentages of the two protonatable substituents. This means that curve bundles can be constructed by varying the degree of initial *N*-acetylation. Thus the initial degree of *N*-acetylation of chitosan has to

be known before further substitution. This eliminates one of the variables and the remaining unknown of interest can be calculated from the experimental EW.
Figure 3.13 illustrates that for a known degree of *N*-acetylation and a specific degree of secondary substitution there exists exactly one EW that can be determined by interaction phenomena. The EW also increases for chitosan with increasing F_A, since the amount of active species decreases and with it the denominator. This decrease in the denominator is more strongly weighted than the decrease in m_0 in the numerator. The latter is due to the acetyl group being lighter than the sodium methylsulphonic acid group.

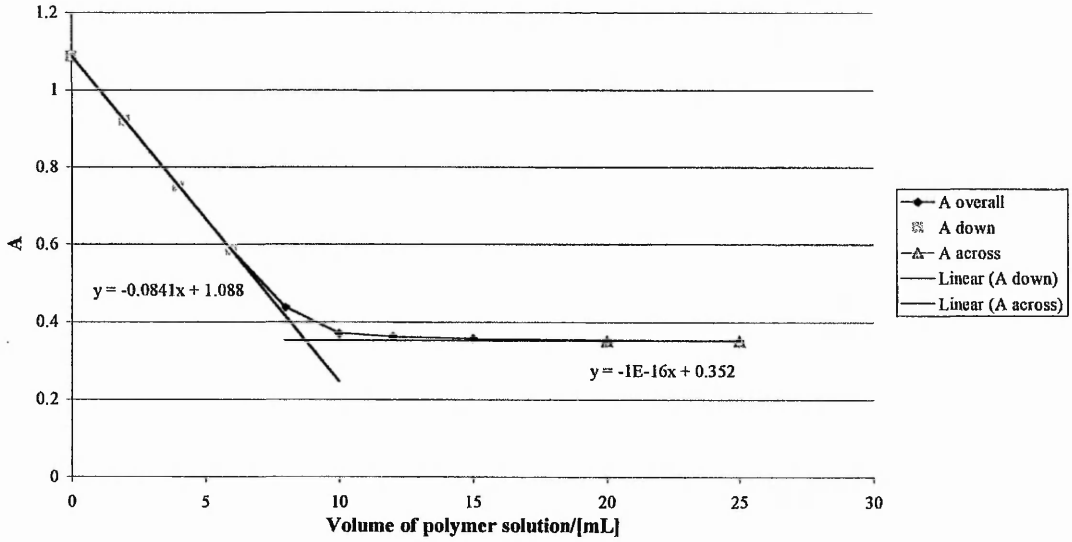
The principle employed for deriving a formula relating EW and degree of substitution is valid and can be applied to more complex systems such as chitosans with residual acetyl groups and secondary partial acylation and tertiary protonatable substitution. For experimental determination of the substitution the degree of achieved derivatisation needs to be determined after each step.

3.2.4 Metachromatic titration

3.2.4.1 Introduction

For the dye-interaction analysis of chitosan derivatives with protonatable secondary substituents a new formula was derived. It takes into account that the substituent is protonatable and thus interacts with the dye ions and the difference in molecular weight of the monomeric units that the secondary substituent would effect (see previous section). From this formula a bundle of curves with suitable starting F_A values can be calculated and plotted with EW *versus* Degree of secondary substitution. From these curves the degree of substitution can be read by selecting the curve with the appropriate F_A and reading the determined EW *versus* Degree of secondary substitution. The equation can be transposed according to x. Thus it is possible to determine the fractions of the amine groups and the secondary protonatable substituent.

Figure 3.14: Metrachromatic titration plot of a typical sample of CHMS (NCm1)



Both the value of the initial fraction of acetyl groups and the EW require to be known. Metachromatic titration or other analytical method such as dye adsorption can determine the latter.

3.2.4.2 Interaction of basic dyes with sulphonate groups

In metachromatic titrations several parameters need to be considered for choosing the concentrations of the interacting dye and polymer solutions:

Beer-Lambert requirements to ensure linearity:

- Absorbances<1.4 for linearity
- Exclusion of aggregation of the dye in the concentration range considered

Titration requirements for accurate graphic and mathematical determination:

- Accurate extinction coefficient of the dye for exact determination of polymer amount at P/D=1 (EP)
- Distinct difference between absorbance of initial dye solution without polymer and the restabilised solutions after the EP
- Polymer concentration and measuring points adjusted to obtain a significant slope through ≥3 data points

The EP of dye to protonatable polymer sites is determined graphically by plotting the titration curve of Absorbance *versus* Polymer concentration and calculating the intercept

point of the regression of the initial slope with the regression of the restabilisation line. Hence it is advantageous to have a steep slope and a restabilisation value differing strongly from the absorbance of the initial dye solution. The first is important ensuring that small variations in the measurement do not cause significant apparent shifts in EP and can be optimized by variation of polymer concentration and choice of measuring points in the first phase of the titration. The second is largely dependent on the specific physico-chemical interaction between the dye and the polymer and was not found to be influenced in a facile manner. Several basic dyes like Basic Blue 9 and Basic Red 2 have been reported as metachromatically active species (180) with anionic polyelectrolytes.

Molar extinction coefficients for Methylene Blue (BB9) have been reported (227) as calculated from literature (228-230). While Shirai reports $\varepsilon=7.1 * 10^4$ L mol^{-1} cm^{-1}, McKays spectrum gives $\varepsilon=7.8 * 10^4$ L mol^{-1} cm^{-1} for solutions in H_2O of Methylene Blue recrystallized from water. The conclusion is that McKay's material was purer than Shirai's. These extinction coefficients cannot be expected to be as accurate as the ones we have been employing for Acid Orange 7, since the dye they have been determined from has not undergone as thorough purification (211). However the coefficients present a valuable indicator of the true value. Further purification of a pre-purified sample provided by the SDC yielded a product with an extinction coefficient of $\varepsilon=7.56 * 10^4$ L mol^{-1} cm^{-1}. The small difference compared to the published value confirms the validity of the latter as a good working coefficient. Inter-dye aggregation phenomena become significant in Methylene Blue at concentrations above 10^{-5} mol L^{-1} (228-230) so that this limits the dye concentration employed in the metachromatic titration, as well as its over three times higher absorptivity, which leads to higher absorbances. The latter however need to be below 1.4 for linearity of Beer-Lambert. Consequently the dye concentration needs to be about three times lower than that of Acid Orange 7. Thus the polymer concentrations need to be adjusted in order to obtain reasonable slopes for the determination of the EP. It has been reported (231), that Methylene Blue starts to aggregate to its dimeric form from concentrations of 10^{-6} to 10^{-4} mol L^{-1}. Looking at the extinction coefficient of Methylene Blue of about 80 000 L mol^{-1} cm^{-1} a suitable concentration would be $1.5 * 10^{-5}$ mol L^{-1} for the final solution concentration ($A \approx 1.2 < 1.4$) and $1.5 * 10^{-4}$ mol L^{-1} (≈ 0.0561 g L^{-1} $= 373.9$ g mol$^{-1} * 1.5 * 10^{-4}$ mol L^{-1}) for the stock dye solution. This would

assure that the dye is present in its monomeric form in solution and gives an absorbance value within the linear range.

The metachromatic titrations carried out on test materials NCm1 and NCm2 converted chitosans of known (0.129 and 0.174) F_A did not show any metachromatic effect due to the interaction of Methylene Blue and the converted chitosan. An approximately constant plot from zero polymer concentration and reaching beyond a calculated EP into the restabilisation area was observed (Figure 3.15). This might be due to the comparatively low polymer concentrations employed, the latter being a direct consequence of the high extinction coefficient of the dye and the fixed path length of 1cm. pH-sensitivity is not an issue for Methylene Blue due to its amine group being quaternary. The pH of the dye-polymer systems in H_2O was determined as neutral. Doubling the dye concentration beyond the point of absorbance linearity did not yield a change in the shape of the plot. A significant time dependence of the reaction was excluded by repeating a measurement of the same solutions after 16h without finding any change. The small coil volume as determined in section 3.3, especially for low F_A material as employed here, might stand in relation to the observed lack of interaction.

Figure 3.15: Metachromatic titration of CHMS with Basic Blue 9

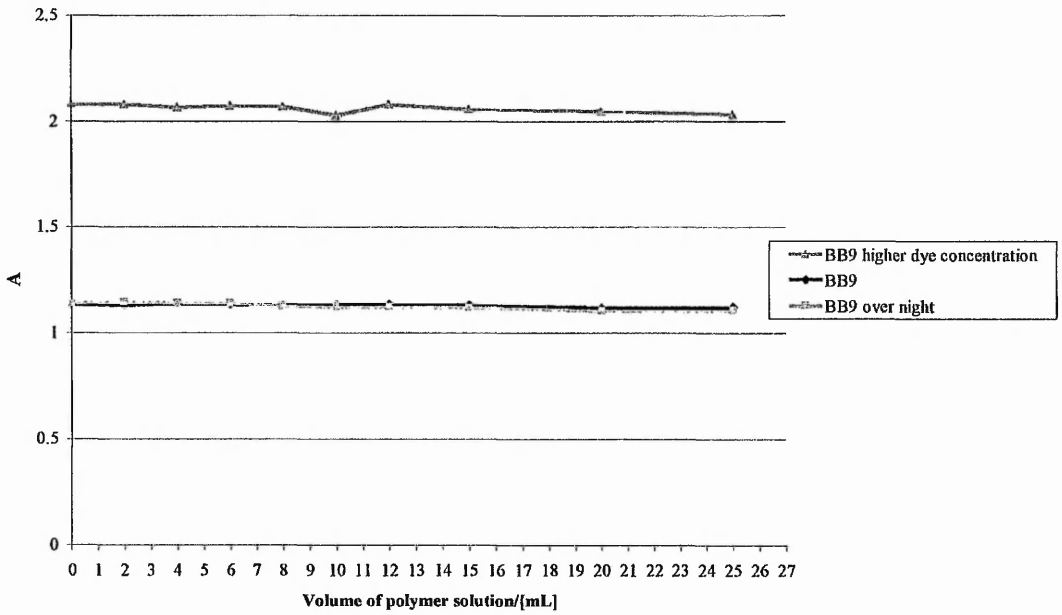
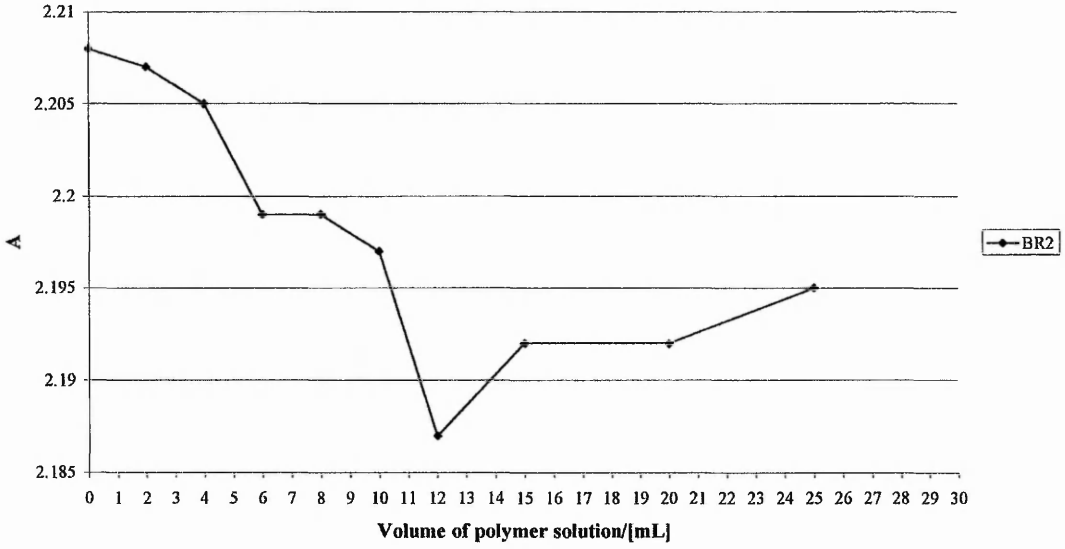
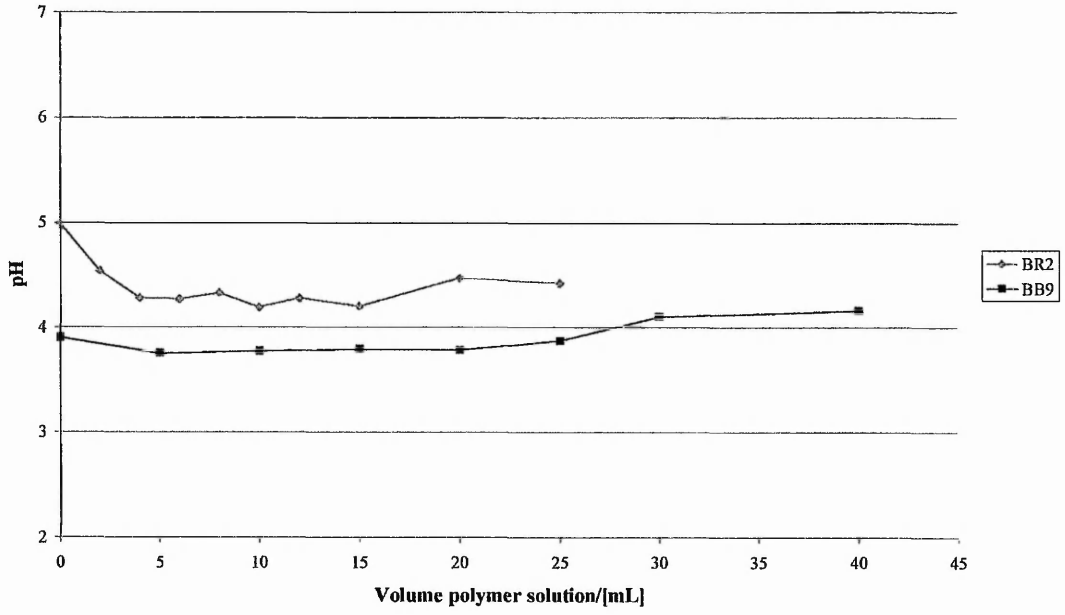


Figure 3.16: Metachromatic titration of CHMS with Basic Red 2



A 4-fold increase in dye and polymer concentration beyond the linearity of Beer-Lambert, followed by a visual assessment of the solutions of equal amounts in sample tubes of the same size against a white background did not show a qualitative metachromatic shift. The solutions were all visually the same.

Figure 3.17: pH over NCm2 concentration in dye solutions



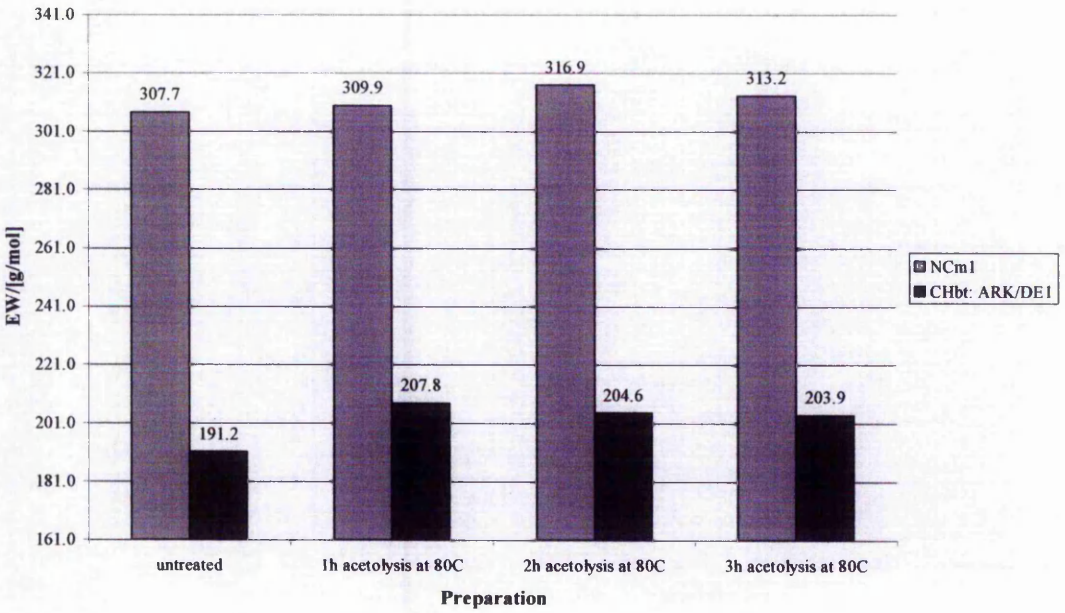
Unpurified BR2 (0.22g/l) showed a weak but clear trend of a metachromatic titration. The effect is not strong enough for quantitative analysis, but shows that there is an interaction of the converted chitosan with metachromatic basic dyes.
When looking at the development of pH in the course of a metachromatic titration, two things become apparent, the systems are slightly acidic and the pH changes in a similar way to absorbance. No obvious explanation was found for this behaviour however it indicates that at least some interaction between the basic dyes and the CHMS has taken place. A closer investigation on the optimal pH of the reaction might yield a means of analysing the polyanion by interaction with cationic dyes.

3.2.4.3 Interaction of Acid Orange 7 with protonated free nitrogen electron pair

Protonating the free electron pair held by the amine nitrogen yields a polymer that holds positive charges and interacts with acid dye showing metachromatic shifts. Since the sulphonic acid group is highly acidic it is not protonated in diluted weak acids such as acetic acid, which means the polymer is present in its zwitterion state. Metachromatic titration was carried out in the same way as for chitosan (3), however making use of EW-substitution for polymer with protonatable secondary substituents relationships as elucidated in chapter 3.2.3.
The results from EW determinations by metachromatic titration suggest, that NCm1 was fully substituted as it exhibited an EW of just over 308 g mol^{-1} which correlates very well with the theoretical value for full substitution at an initial F_A of [0.13] of EW=307 g mol^{-1}. (Plot see section 3.2.4.1 Figure 3.14.) The EW of the initial chitosan had previously been determined as 191 g mol^{-1} (hence F_A=[0.13]) by metachromatic titration. Further metachromatic titrations were carried out with samples that were acetolysed (hydrolysed with acetic acid) for 1, 2 and 3 hours at 80°C in 0.5M HOAc. These samples gave a successful metachromatic titration with Acid Orange 7 and showed full substitution of the NCm1 utilising the novel EW-substitution relationship (3.2.3) confirming it to be a suitable tool for the determination of the degree of sodium *N*-methylsulphonation. The idea of confirmation of the results by titration of the same polymer with prior acetolysis was that the weight of material would be counted including the substituents, while the amine groups would be free after the hydrolysis to interact with the dye anions as recovered chitosan. The EW resulting however would be that corresponding to the substituted material, since that was what was weighed into the polymer solution used in the metachromatic titration.

A slight trend can be see in both the CHMS (sample NCm1) and the chitosan of which NCm1 was prepared, treated under the same conditions. The trend shows first a very slight increase and then a decrease in EW with increasing acetolysis time. Minimal *N*-acetylation of the materials, as is possible under these conditions (232), could provide an explanation for this trend further confirming that metachomatic titration as reported for chitin and chitosan (3) is a satisfactory method for the determination of the EW of CHMS without prior acetolysis of the substituent being necessary.

Figure 3.18: EW determined for varying preparation conditions of the sample



3.2.5 Dye adsorption

Dye adsorption as an easy low cost technique requiring very little operator time was considered for determining the substitution degree for routine analysis of CHMS materials. A chitosan of known F_A [0.129] was converted to CHMS by reaction of never-dried material. The material's particle size had been decreased to ensure facile and complete reaction by shearing in a *Waring* commercial blender. The actual EW of fully substituted CHMS at the respective F_A was determined as 307g mol^{-1} by employing the equations elucidated in section 3.2.3. Unlike chitosan, CHMS is soluble in H_2O. A requirement for interaction with acid dye to take place is the protonation of the free nitrogen electron pair.

First tests showed that dye uptake as used for chitin and chitosan in 0.1M HOAc as a solvent yielded a too high EW. This was put down to dissolution of fractions of the material in the dye solution; the bulky dye ions at the concentrations employed were not sufficient to prevent partial dissolution. It becomes clear that on heating in 0.1M HOAc/Acid Orange 7 solutions at 60°C and over t>16h, and approximately 24 hours in this case, the secondary substituent does not become quantitatively hydrolysed. This means that chitosan is only partially recovered for interaction and reprecipitation/adsorption in the presence of the dye counter ions. Dissolution-reprecipitation was also backed by the fact that the dye-polymer composite yielded a very fine residue, which could not be completely removed by filtration through glass wool. The latter may have contributed to diffuse adsorption of dye, which would help to counteract the too high EW obtained.

In order to avoid the loss of interacting material through dissolution various solvent systems were tested for suitability. The adsorption medium was rendered less solvating by exchanging varying proportions of the aqueous medium with 0.1M HOAc in MeOH. The latter makes the solvent system less polar without itself being too bulky and causing major obstruction to diffusion into the particle. The tested proportions were 100/0, 50/50 and 25/75 0.1M HOAc/MeOH. Another consideration, namely to introduce bulky cations like tetraethyl ammonium ions, was not carried out since the bulky cations might interact with the dye itself.

The EW values obtained are slightly too high for the material in 100% 0.1M HOAc and twice as high for the material in 50/50 v/v MeOH/0.1M HOAc, while the material in 75/25 v/v MeOH/0.1M HOAc did not adsorb any dye at all. It becomes clear that on heating in 0.1M HOAc/Acid Orange 7 solutions at 60°C and over t>16h and approximately 24 hours in this case the secondary substituent does not become quantitatively hydrolysed. This would explain the sludgy appearance of the residue as formed by equilibrium of dissolution of the NCm1, which then readily undergoes substituent hydrolysis in homogeneous medium, and reprecipitation as the initial chitosan is regenerated. The EW value of the material in 50/50 v/v MeOH/0.1M HOAc is twice as high as theoretically possible, which suggests, that it has not adsorbed the dye despite its dark red appearance, which is due to a high polymer/dye concentration on the surface. This could be caused by inaccessibility of the active sites to the dye ions, which is supported by the fact that the

particles were only slightly flexible and obviously only little swelling had occurred in the present medium. The particles, when cut into half consisted of a very dark red and only very slightly swollen outer shell. The particle appeared to have been radially expanded (i.e. "blown up") and a lighter coloured inner core, which consisted of orange "sludge" with a very similar appearance to the residue in the non-methanolic adsorption. On drying the particles the former lighter sludge dried to a similarly dark-appearing material, while the smooth globular hollow inside the particle became even more apparent. The expansion of the particles could have been caused by osmotic pressure building up within them due to material dissolving with solution-entropy being the driving force. While on the outer surface of the particle the polymer interacts with the dye ions in higher dye concentration and is not swollen, so that dye ions cannot diffuse into the material. Obviously the polymer will not dissolve under these conditions, so metachromatic interactions of the polymer and the dye can be excluded.

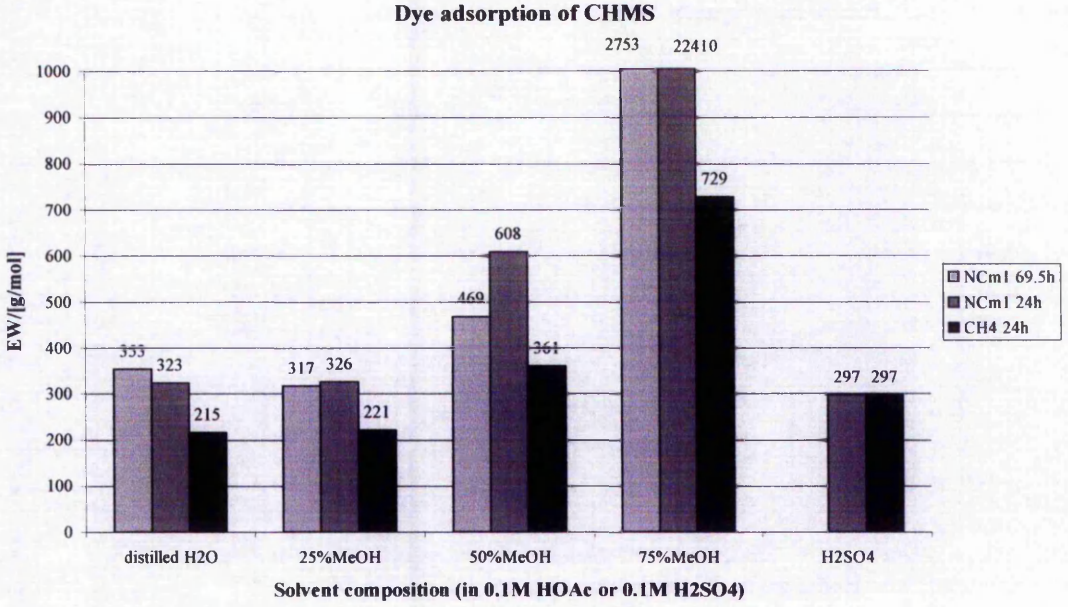
A further observation was made on making up the methanolic dye solutions. The solutions were visually more orange than the solution of the same concentration in 0.1M HOAc. This is a consequence of the difference in polarity of the solvents employed, which changes the energy state of the dye molecules with increasing MeOH concentration compared to the dye molecule in 0.1M HOAc. In further tests the NCm1 will undergo dye adsorption analysis under the following conditions:

0.1M H_2SO_4 was considered as a stronger acid compared to 0.1M HOAc while at the same time not binding to the amine like HCl and leaving it freely protonated for interaction. A possible problem would be degradation of the polymer and loss of adsorbing material. As a comparison the dye uptake behaviour of chitosan (CHbt ARK/DE1) in the different media was tested. The effect of longer adsorption times on the diffusion behaviour into the particle was studied. As can be seen in Figure 3.19 adsorption in 0.1M H_2SO_4 yielded a too low EW which can be put down to chain degradation and loss of material taking part in adsorption.

Longer adsorption times of 3 days improved the diffusion into the particle, as shown by decreasing EW compared to 24h adsorption time. Comparing the adsorption studies on chitosan and on CHMS it can be concluded that MeOH is increasingly hindering the diffusion of the dye into the polymer particle and thus causing too high an apparent EW. Looking at the values of EW for CHMS between 0% MeOH and 50% MeOH there appears to be a minimum for EW, which could represent the value for optimum MeOH

concentration. This minimum can easily be explained by the fact that 2 counteracting phenomena are effective here, namely diffusion into the particle on one side and dissolution in the medium on the other. The exact determination of an optimum MeOH concentration for dye adsorption was not undertaken since results from metachromatic titration suggested a suitable method, which was less solvent intensive.

Figure 3.19: Apparent EW determined by dye adsorption of NCm1 with increasing MeOH concentration at an HOAc concentration of 0.1M and in 0.1M H_2SO_4



3.2.6 Solubility and degree of substitution of CHMS

Water solubility can be used as a test for conversion of chitosan to fully *N*-substituted CHMS for several reasons. CHMS of fractions of re-*N*-acetylation as high as 0.61 are water soluble with a resulting degree of ionisable substitution of 0.39, which is comparatively low. This also shows the high solubilising quality of the sulphonic acid group in this case. The fact that the reaction was carried out under heterogenous conditions is another indicator that the reaction has to be complete in order to obtain water solubility, since otherwise water insoluble block copolymers would be retained in the centre of reacting particles. Chitin only becomes soluble at degrees of de-*N*-acetylation of about 60% and above (12) p 64 for heterogeneous de-*N*-acetylation, which equates to a concentration of protonatable groups of ≥ 60%. Fully substituted CHMS with an initial F_A

of 61% however only possesses a concentration of water soluble sulphonic acid groups of 39% when fully substituted. The clearest point is the result obtained by metachromatic titration for a heterogeneously reacted material CHMS-M [0.148] which had a starting F_A of [0.148] and was prepared from a medium molecular weight fraction. The material although insoluble in water was solubilised in 0.1M HOAc and yielded a degree of overall substitution of 94% by metachromatic titration. Thus a residual amine concentration of 6% rendered the material insoluble in H_2O at a concentration of sulphonic acid groups of 79%. The material was only highly swollen in water although soluble in 0.1M HOAc. This proves that testing the solubility of the CHMS material once dried can be used as an assay for the completeness of substitution with very good approximation.

3.2.7 Summary

- Fully substituted CHMS was prepared by dissolution-precipitation of crude and re-*N*-acetylated chitosan as well as from never-dried chitosan
- A gravimetric assay was developed for assessing substitution of CHMS
- The sodium *N*-methylsulphonation reaction being quantitative was confirmed by gravimetric analysis of a fine particular sample of CHMS (N8) prepared and isolated by dissolution-reprecipitation by a co-worker
- EW-substitution relationships valid for complex chitosan derivatives with protonatable secondary substituents were derived through mathematical manipulations and applying a principle established for chitin and chitosan copolymers. The principle of derivation can be employed for determining substitution of chitosan derivatives of branched complexity given that the EW can be determined and was determined after each addition of a sustituent to an idealised chitosan [0.00] model.
- The novel formulae was successfully employed for the determination of protonatable secondary substitution in CHMS with known initial (primary substitution) F_A thus confirming its validity.
- A facile metachromatic assay of protonated CHMS with Acid Orange 7 for determination of EW, and ultimately the degree of substitution, was developed and confirmed by comparison with regeneration of the initial chitosan for direct dye-interaction
- The use of basic dyes such as BB9 and BR2 for direct metachromatic titration of the sulphonic acid group in CHMS was found to be unfeasible. Further optimisation of analytical parameters like e.g. pH could lead to more favourable outcomes
- A dye adsorption assay of CHMS with Acid Orange 7 in methanolic acetic acid systems showed promise
- Solubility was established as an assay to ascertain qualitatively the extent of sodium *N*-methylsulphonation of chitosan

3.3 Determination of Mark-Houwink relationships of CH and CHMS for viscometric determination of molecular weight

3.3.1 Introduction

Next to the degree and distribution of *N*-acetylation, the degree of polymerisation is one of the most important structural characteristics of chitin and chitosan. For comparability and reproducibility of results on different chitosan samples it is necessary to be able to specify the nature of the material to which the data and observations apply, molecular weight being one crucial, determining factor in the polymer behaviour.

Sophisticated methods have been used for the determination of molecular weight such as size exclusion chromatography SEC (GPC), SEC-MALLS (Multi-angle laser light scattering) and Multi detector SEC.

The use of SEC for chitosan with dextran standards for secondary calibration is of limited feasibility, since dextrans do not elute at the same time as chitosan due to their differing hydrodynamic volume (193) and adsorption and deposition of the polycation onto the column. It has been shown in a comprehensive review (193) of instrumental methods for the determination of DP and DPD that SEC-MALLS is a suitable method for the determination of accurate molecular weight data for chitosans with molecular weight ≥ 100 000. Some inaccuracy occurs due to some adsorption and deposition of the cationic polymer onto the column, whilst in SEC the separation relies on pure physical separation by size and thus possible distance travelled though the column. Adsorption onto the column is particularly pronounced for cationic polymers e.g. chitosan (196). Since SEC-MALLS involves expensive equipment a more routine method to determine molecular weight and molecular weight distribution was sought. Determination of the limiting viscosity number can be carried out using comparatively simple equipment with suspended level viscometers in a temperature controlled water bath. From the limiting viscosity number the molecular weight can be determined by employing the Mark-Houwink relationship.

$$MW = \sqrt[\alpha]{\frac{LVN}{k}}$$

$$LVN = k * MW^{\alpha}$$

k and α values are generally specific to a given polymer/solvent system at a given temperature and have to be determined experimentally from the limiting viscosity number values of materials with a molecular weight and molecular weight distribution known from an absolute technique such as e.g. SEC-MALLS, end group titration or analytical ultracentrifugation.

Extensive work has been done on finding accurate k and α values for chitosan, but evaluation of the respective values has lead to the conclusion that satisfactory values have not been obtained (195), (158). The constants failed to give a constant DP for materials only varying in their F_A and the values themselves were not in agreement with values obtained from ultracentrifugation. It was concluded that further study is required.
The philosophy of the present investigation was the fact that a non-degradative process was found (142) to convert chitosan into a water-soluble entity by sodium *N*-methylsulphonation of the same DP as the initial chitosan. The material provides a crucial advantage for molecular weight determination compared to chitosan. Unlike chitosan it exists as a polyanion in neutral medium. Using a conventional negatively charged column material would thus counteract adsorption of material onto the column. This is favourable for the accurate determination of molecular weight and molecular weight distribution by SEC-MALLS as an absolute method.
A 2D matrix of chitosans was prepared using one sample of initial chitosan. The two dimensions of the matrix were molecular weight and F_A. From the initial material 3 different fractions of molecular weight were prepared by chain scission with nitrous acid (215). Portions of the three different fractions were re-*N*-acetylated to yield 5 different discreet values of F_A. The limiting viscosity number values of the chitosan matrix were determined. From the produced chitosans another 2D matrix of materials was produced by quantitative sodium *N*-methylsulphonation under non-degradative conditions. These materials could now be analysed according to their limiting viscosity number by dilute viscosity measurements and their DP and their DP retention across varying F_A by SEC-MALLS. From the obtained DP and DPD values statements about k and α relationship for CHMS as well as for chitosan could be made.

3.3.2 Preparation and characterisation of initial chitosan for preparation of a 2D F_A-MWD sample matrix

3.3.2.1 Preparation of the chitosan matrix

a) Preparation of chitosan from squid pen

For the preparation of chitosan with a high initial DP squid pen was considered, since this very accessible β-chitin had been converted under mild conditions to chitosan of very high viscosities (219). The hammer milled squid pen was deproteinated and decalcified prior to deactylation. The product was strongly discoloured and showed a very low viscosity. Severe chain degradation had occurred despite the fact that the reaction process used is favourable due to the limitation of oxygen. It has been shown that the presence of oxygen increases the degradation of the polymer chain by alkali (233). An explanation for high level of depolymerisation could be acid hydrolysis during the decalcification step. The fact that the pH did not rise during decalcification indicates that 2N HCl was excessive to bring about decalcification in this very accessible and low calcium carbonate material. Unlike chitosan, which is better protected against acid hydrolysis by charge repulsion, chitin's glycosidic link is very susceptible to acid hydrolysis (12). Another possible source for chain degradation is microbial degradation of the initial native material while being stored at room temperature and ambient humidity. It has been observed that a chitosan prepared after longer storage of the material did not show as high viscosity (400 cps, 1% solution in 0.1M HOAc) as the earlier material (2400 cps, 1% solution in 0.1M HOAc) (219). A very likely origin for the present degradation are oxidative processes during the three deactylation steps especially the last one, since its parameters were the severest. Overheating of the reaction system despite vigorous agitation on the hot plate may have occurred. This is reflected in the severe browning of the material.
For the scope of producing high molecular weight highly de-*N*-acetylated chitosan a high molecular weight commercial sample was chosen and further de-*N*-acetylated.

b) Further de-N-acetylation of a commercial chitosan sample

The initial material for the preparation of the 2D FA-MWD sample matrix was prepared by further de-*N*-acetylation of a commercial chitosan sample CHb (see Experimental) under

non-oxidative conditions. The F_A was decreased from [0.28] to [0.15] and the yield was 77% overall of purified chitosan with an initial bulk viscosity of 230 cps (1% solution in 0.1M HOAc at 17.5°C).

c) Degradation of a chitosan sample with nitrous acid to three different fractions of molecular weight

Three different fractions of the initial chitosans varying only in their molecular weight and molecular weight distribution were prepared by chain degradation with nitrous acid (215) at polymer and acid concentrations of 1% and 0.1M unless otherwise stated. The depolymerisation can be assumed random since the reaction kinetics have been found independent of molecular weight (215). GPC-LALLS (gel permeation – low angle laser light scattering) experiments for materials degraded by the above method have indicated random distribution and a weight average molecular weight over number average molecular weight approximating a value of 2 has been reported (165). The degradation was monitored qualitatively quasi-online by measuring the bulk viscosity over time with a *Brookfield Synchro-Lectric LVF* viscometer. This allowed the production of qualitatively different molecular weight fractions as well as the determination of the end point of reaction. The initial material* was divided into 3 portions of which one portion was kept "as is", i.e. undegraded to give the high molecular weight fraction (H) and the two remaining thirds were degraded to a medium molecular weight (M) and to a low molecular weight fraction (L). The latter was degraded in two steps, since the determination of the limiting viscosity number yielded very similar results for M and L despite the difference in bulk viscosity.

* Degrading the initial material at low F_A rather than different fractions of chemical composition after reacetylation further ensures repeatability of molecular weight distribution along one value of F_A apart from the obvious practical advantages. The reaction only attacks the glucosamine unit in the course of diazotation and thus materials with different F_A have different kinetics and speed of reaction due to different concentration of reactive sites (215). High values of block-type acetyl group distribution, where they are present can compromise randomness of reaction and thus homogeneity in acetamido unit distribution has to be insured for the generation of Bernoullian molecular weight distributions.

Table 3.13: Viscosity changes as a result of chitosan chain scission by nitrous acid

MW fraction	H	M	L
Initial bulk viscosity/[cps]*	230 RT=17.5°C	150 RT=22°C	100 RT=23°C
Initial LVN/[mL/g]	771	771	771
Bulk viscosity after chain scission with nitrous acid	-	75	60
LVN after chain scission/[mL/g]	-	465	461
Bulk viscosity (2% polymer) before further chain scission	-	-	69 RT=22.5°C
Bulk viscosity after further chain scission	-	-	34.5
LVN after further chain scission/[mL/g]	-	-	414

d) Reproducible re-N-acetylation of chitosan samples across different degrees of polymerisation

Great attention was paid to making the overall process from initial chitosan to corresponding pairs of chitosan/condensation product

- further de-*N*-acetylation of initial chitosan (single lot)
- production of varying molecular weights
- re-*N*-acetylation
- conversion to the condensation product CHMS

as systematic and reproducible as possible. The aim was to obtain materials with the same DP distributions along the range of varying degrees of *N*-acetylation and congruent degrees of *N*-acetylation across the three molecular weight fractions. Thus the process will be

* At this point the material is still identical for all three prospective molecular weight fractions.

described in great detail to make the nature of the matrix of materials obtained as comprehensible as possible.

Accurate weights of the respective chitosan samples were weighed into tared glass dye pots. The polymer was wetted out with a small amount of 0.1M HOAc on stirring in order to ensure good dispersion. The amount of 0.1M HOAc was then adjusted by weight to 100mL/g chitosan. The vessels were sealed and left to stir in a temperature conditioned laboratory (20 ± 2 °C) for a standardised time, which had been determined by the time it took for the high molecular weight fraction to completely dissolve. After dissolution the solutions were diluted by weight with MeOH (100mL/g chitosan minus 50mL for reaction mixture). The reactant acetic anhydride was freshly prepared to a stock reagent mix of 7.15mL/250mL in a volumetric flask. Of this mixture aliquots depending on the target degree of *N*-acetylation were weighed into measuring cylinders, which were filled up with MeOH to 40mL. The weight of the added diluted reaction mixture was determined for the first system of each target substitution and subsequently the dilution was carried out by weight. The diluted reaction mixture was added to the polymer solution under vigorous stirring. The reagent residue remaining in the cylinder was rinsed with 10mL MeOH by weight and added to the reaction system whilst homogenising it. The reaction vessel was put into a viscometer bath set accurately at 25°C and kept at this temperature for 3.5h. This reaction time was chosen, since butyrylation of chitosan film has been found to be nearly completed (133) after this time and homogeneous reaction with the more reactive acetic anhydride can be expected to be even more facile reaction. The reaction was stopped by reprecipitation with methanolic ammonia (54.90g NH_4OH /190.8g MeOH) 17.42g/g chitosan.

The materials were prepared in sample triplets, re-*N*-acetylating one sample of each molecular weight fraction H (high MW), M (medium MW) and L (low MW) to the respective degree of *N*-acetylation.

The amounts of anhydride required were obtained from a series of samples prepared and analysed by a co-worker from a sample with initial F_A [0.085]. The degree of *N*-acetylation achieved using a specific batch of anhydride was plotted *versus* the amount of anhydride employed. A correlation (approximation) equation was calculated from Microsoft® Excel 97 SR-1 from which the amount of anhydride necessary to achieve a certain F_A was approximated from consideration of initial F_A (results see Table 3.14).

Figure 3.20: Reaction efficiency of re-*N*-acetylation of 2.5g chitosan [0.085] with a specific lot of acetic anhydride

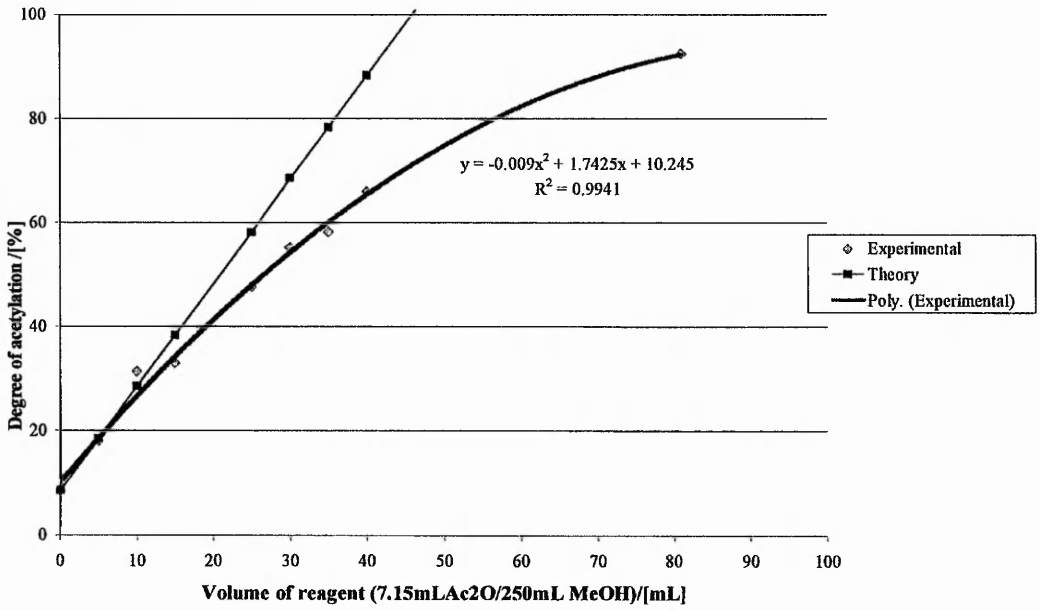


Table 3.14: Target amounts of anhydride for F_As aimed for

F_A target	Chitosan/[g]	Target amount of reagent mix/[mL]
0.11	3.00	0
0.25	2.00	5.1
0.35	3.00	14.5
0.45	2.00	15.5
0.65	3.00	46.6

e) Processing conditions for re-N-acetylating chitosan samples

In the overall process great care was taken that the respective molecular weight fraction was kept separately from any of the others and that cross contamination through tools and apparatus did not occur.

After reaction as described in detail in the previous section the polymers were washed to neutral according to litmus paper with MeOH* with isolation by centrifugation to minimise material loss as compared to conventional filtration. The centrifugation was carried out by combining the respective fractions in two vessels each in 2 steps and centrifuging (4200-4300 revs/min) in 30-minute intervals, with the low molecular weight fraction starting off with 60 minutes in order to ensure retention of the low molecular weight material. Once the combined material was compacted the first washing solvent was added. The neutral polymer fractions were kept in MeOH under inert conditions until they underwent further processing, typically the next day.

Table 3.15: Process conditions and times and their variations in the preparation of the 2D chitosan matrix

Process	**Temp./[°C]**	**Duration**	
Dissolution of chitosan	20.0±2	17.5±0.5h	
Dilution with MeOH	20±5	up to 20min	
Mixing of reagent mix	20±5	up to 8 min	
Dilution and addition of reagent mix	20±5	up to 20 min	
Reaction and subsequent precipitation	25.0±0.1	3.5h±10min	
Addition of first washing solvent after	20±5	2h±0.5h	
Centrifugation and neutrality achieved after alkaline precipitation	36 (centrifuge, spot check) 20±5	4h±2min* 6h45min±7min**	5h±1h15 min***

* For 2.00g chitosan

** For triplet [0.25] as it was washed to neutrality with water instead of MeOH, which is more time intensive due to the precipitate remaining highly swollen throughout

*** For 3.00g chitosan

* Except for fraction 0.25, which was washed to neutral with H_2O and then solvent exchanged with MeOH

Table 3.16: Process amounts and their variations in the preparation of the 2D chitosan matrix

Substance	Amounts	
Chitosan	2.00±0.01g	3.00±0.01g
0.1M HOAc	198.90±0.08g	298.35±3g
MeOH	117.39±0.00	195.65±0.01g***
Reagent mix	target amount ± n.a.*	target amount ± 0.00
MeOH for dilution	target amount ± n.a.*	target amount ± 0.00
Additional MeOH for quantitative transferral of reagent solution	7.45±0.03g	7.45±0.03g exception for [0.65]: 4.07±0.00g**
NH_4OH/MeOH (36.60g/127.20g)	34.84±0.09g	52.26±0.16g

* data loss for triplet [0.25]

** reagent mix volume exceeded the 40 mL mark without further dilution, so only 5mL were employed for rinsing

*** except: H[0.35] (±19.60g) and L[0.65] (±1.82g)

The material's wet bulk weight was determined by filtering into a Buchner funnel through a sandwich filter. The latter consisted of a paper filter sandwiched between two layers of polyester monofilament mesh and was used to prevent material loss in the low molecular weight range. The chitosan was divided into two equal portions of which one was kept as never dried chitosan in MeOH for further reaction to give the condensation compound and the other was solvent exchanged with diethyl ether for air drying.

From the latter the yield, degree of *N*-acetylation and the limiting viscosity number of the chitosan were determined.

As can be seen in the above two Tables 3.15 and 3.16 the reaction conditions involved only a very small variation in any one parameter at a time, reflecting the crucial considerations for the preparation of samples with defined F_A, and molecular weight and molecular weight distribution respectively.

3.3.2.2 Characterisation of the chitosan matrix concerning F_A

The F_A values of the material of the chitosan matrix were determined by dye adsorption with Acid Orange 7. The relative accuracy between the values across the range of three different degrees of *N*-acetylation is typically better than 5%, which is well within the accuracy of the method. The value of relative accuracy for the [0.148] materials is comparatively high, which is due to it being the lowest F_A and thus small absolute variations amount to comparatively large relative variations. When looking at the absolute deviation from the mean value it compares very well to the other values, which are typically below a variation of 3% in degree of *N*-acetylation, which also is very well within the method's accuracy. Thus it can be concluded that the materials show uniformity in their degree of *N*-acetylation across a triplet of different molecular weight values with good accuracy.

Table 3.17: Degree of *N*-acetylation of the samples of the chitosan matrix as determined by dye analysis

Sample	F_A	Mean F_A	Relative accuracy/[%]	Absolute accuracy	Yield/ [%] theory
H [0.148]	0.1715	0.148	18.1	0.027	0.91
M [0.148]	0.1214				0.93
L [0.148]	0.1514				0.97
H [0.280]	0.2854	0.280	4.9	0.014	0.98
M [0.280]	0.2880				0.92
L [0.280]	0.2661				1.00
H [0.348]	0.3482	0.348	1.6	0.006	0.95
M [0.348]	0.3538				0.94
L [0.348]	0.3421				1.00
H [0.459]	0.4280	0.459	4.5	0.022	1.01
M [0.459]	0.4809				0.96
L [0.459]	0.4682				0.91
H [0.609]	0.5937	0.609	2.1	0.013	1.01
M [0.609]	0.6223				0.96
L [0.609]	0.6109				1.01

Underestimation of the initial F_A as well as steric considerations during the progress of the reaction, since initial residual *N*-acetylation is mostly block-type, while homogeneous re-*N*-acetylation gives random distribution of substituents along the polymer chain (30), lead to variation from the curve of *N*-Acetylation *versus* Volume of anhydride used. For chitosan triplet with a target F_A of [0.65] the value [0.609] is lower than expected, which might be due to incomplete transferral of reagent from the measuring vessel. The latter was a consequence of the larger initial reagent volume and consequently smaller remaining rinsing MeOH volume. A strong indicator that random *N*-acetylation is actually achieved is the fact that products with a F_A of > [0.65] are still soluble, while chitosan produced by heterogeneous de-*N*-acetylation of chitin, thus containing block copolymer (234), only becomes soluble at F_A values around ≤ [0.4] (12). Further indication is a comparison of chitosans prepared by homogenous re-*N*-acetylation and heterogeneous de-*N*-acetylation by NMR spectroscopy of which the acid soluble fractions showed random Bernoullian distribution (176). However more significant confirmation might be obtained by AFM (atomic force microscopy) imaging (235).

3.3.3 Preparation and characterisation of water-soluble amphiphylic CHMS juxtaposed to the 2D chitosan matrix

3.3.3.1. Preparation of a CHMS matrix juxtaposed to the chitosan matrix

CHMS samples were prepared by heterogeneous sodium *N*-methylsulphonation of the samples of the chitosan matrix conveniently stored as never-dried material in MeOH as described in Experimental. Small samples were taken to test for solubility and were blotted dry on filter paper before dissolution in H_2O. Since after a reaction time of 19.5h all the samples proved soluble in H_2O the bulk of the polymer was recovered by centrifugation from the reaction system and purified by exhaustive washing to remove by-products and unreacted reagent while at the same time leaving the derivative quantitatively undissolved. This was achieved by washing with 75% MeOH or EtOH. After drying the materials subsequent to recovery it was found that some of the bulk samples' solubility was incomplete and that test samples need to be completely dry and unswollen to make the test for solubility of the products reliable.

Table 3.18: Solubility in H_2O overnight of converted matrix products after first step conversion

FA/MW fractions	CHMS-H	CHMS-M	CHMS-L
0.148	-	- (very highly swollen particles)	- (cloudy suspension with fine particles)
0.280	-	- (cloudy suspension with fine particles)	-
0.348	- (very highly swollen particles, near invisible)	+	+
0.459	+	+	+
0.609	+	+	+

- =not soluble

+ =soluble

Table 3.19: Solublity in dilute HOAc on stirring of products found water-insoluble

FA/MW fractions	CHMS-H	CHMS-M	CHMS-L
0.148	- 2 days	+	+
0.280	+	+	+
0.348	- over 3 days		

- =not soluble

+ =soluble

As can be seen in Table 3.18 the reaction was incomplete for samples with a low degree of *N*-acetylation and thus high requirement of secondary substitution. Only two samples of CHMS could not be solubilised by protonation of residual amine groups in dilute HOAc:

CHMS-H[0.148] and [0.348]. Of the initially water-insoluble derivatives CHMS-L[0.148] and CHMS-M[0.280], representing the most finely disperse material, could be solubilised by repeating the heterogeneous reaction. The remaining materials CHMS-M[0.148], CHMS-H[0.148],CHMS-L[0.280] and CHMS-H[0.348] could not be converted to water-soluble CHMS by heterogeneous reaction with decrease of particle size by cutting, by increase of water concentration in the solvent to increase swelling, nor by the homogenising reaction as the material resisted complexation and dissolution with FSBS in H_2O unlike unreacted chitosan.

The latter is put down to strong inter chain interaction formed on drying the incompletely reacted material. Thus water-soluble materials CHMS-M[0.148], CHMS-H[0.148],CHMS-L[0.280] and CHMS-H[0.348], required to complete the matrix, were produced by homogenising reaction of dried initial chitosans M[0.148], H[0.148],L[0.280] and H[0.348]. The yields as seen in Table 3.20 show no trend along different molecular weights, which suggests that the molecular weight distribution was retained during product recovery.

Table 3.20: Yields of matrix CHMS produced by homogenising reaction

Derivative	Yield/[%]
CHMS-H[0.148]	85
CHMS-M[0.148]	86
CHMS-L[0.280]	84
CHMS-H[0.348]	92

3.3.3.2 Sample characterisation concerning their degree of substitution

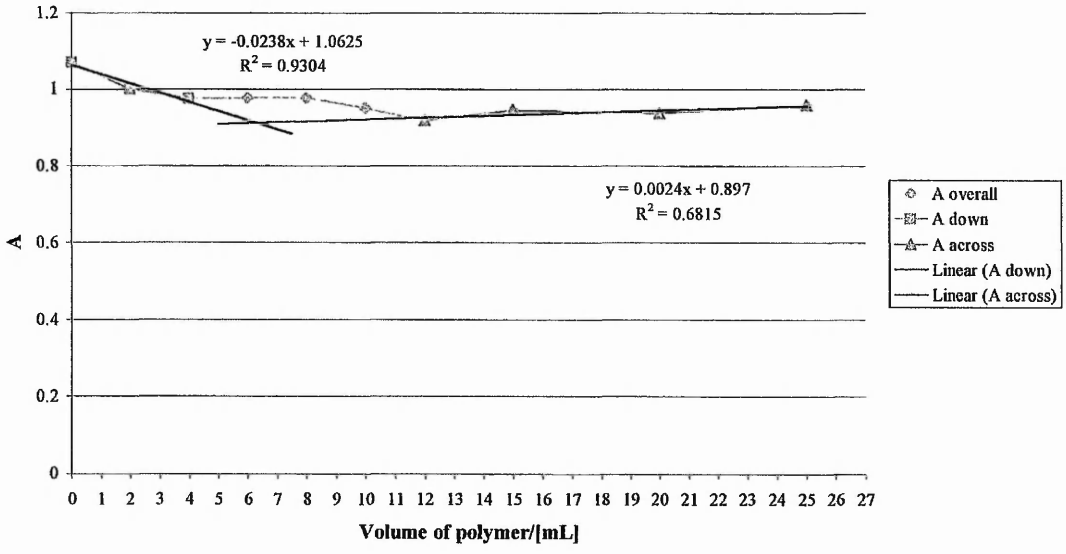
The CHMS materials were characterised for substitution by metachromatic titration in the protonated form in 0.1M HOAc with Acid Orange 7.

Table 3.21: Degree of substitution of CHMS matrix samples

	D_S/[%]		
F_A	CHMS-H	CHMS-M	CHMS-L
0.148	101	111	102
0.280	100	103	97
0.348	103	98	100
0.459	89	82	89
0.609	88	99	98

The samples of F_A-fractions [0.148], [0.280] and [0.348] clearly show full *N*-substitution of the material. The variation around the 100% value, and especially the theoretically impossible values above this, are within the accuracy of the method and confirm a statistical variation around a true value of 100%. For fractions [0.459] and [0.609] the results are getting somewhat less reliable due to changes in chain conformation as further discussed in the following sections and subsequent decrease in dye-polymer interaction. Another effect that causes seemingly too low

Figure 3.21: Metachromatic titration plot of CHMS-M[0.609]



values of substitution is the lower charge density in the coil, which also decreases the metachromatic interaction of the dye with the polymer. This leads to the graphical problem of less sharp intersection angles and thus higher inaccuracies in the determination of EP. It becomes clearly visible for [0.609] materials. A typical example of a metachomatic titration plot can be seen in Figure 3.21.

Materials [0.459] and [0.609] are around the F_A value for water-soluble chitosan. This could mean that they are generally more highy swollen, which would lead to an overall more uniform heterogeneous reaction with FSBS. *N*-Carboxymethyl chitosan, which has got a very similar structure, can be prepared in a homogeneous reaction and becomes water-soluble at a degree of substitution of around 50%. This could suggest, that CHMS materials [0.459] and [0.609] may have become water-soluble at an overall degree of substitution <100% due to a more uniform reaction, which seems crucial for solubility. Since water-solubility was used as a criterion for the completeness of reaction it is possible that the reaction was stopped when solubility was reached, however before fully substituted material was achieved for materials [0.459] and [0.609].

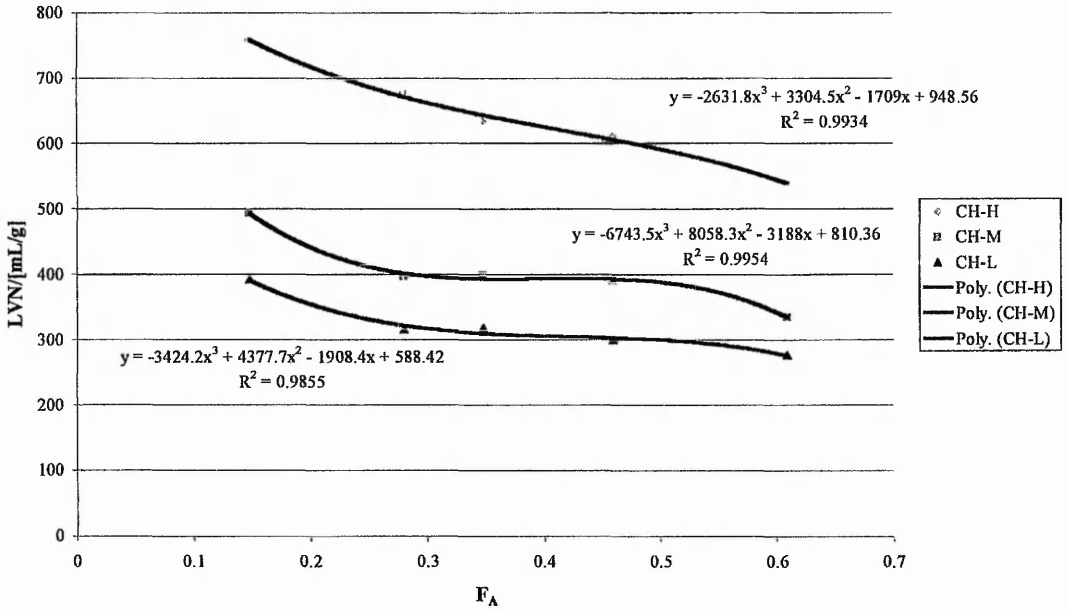
3.3.4 Determination of the the limiting viscosity number values of the chitosan and the corresponding CHMS matrix materials

3.3.4.1 Dilute solution viscosity and the limiting viscosity number values of the chitosan matrix

Yields of the chitosan matrix as to be seen in Table 3.17 were typically around 95% with no obvious trend of material loss related to molecular weight. This indicates that material loss was representative of the molecular weight distribution and not loss of just the low molecular weight fraction. Values of over 100% were calculated which is theoretically not possible and is a result of inaccuracy in the determination carried out by taking wet bulk samples and subsequent drying for yield determination. It has been shown that under the mild conditions of re-*N*-acetylation employed the DP of chitosan is largely retained (195). Also 0.1M HOAc/0.2M NaCl had been used as a suitable solvent for the limiting viscosity number determination of chitosan at 25°C (203). It can be seen in Figure 3.22 that the limiting viscosity number decreases with increase in F_A. The amount of sugar units and thus of polymer chains in a given volume of solution decreases with an increase in average weight of the sugar unit m_0 as a consequence of substitution of the amine group, thus the

originally measured Limiting viscosity number values were plotted *versus* Polymer concentration/[g/mL] corrected with a factor $m_0[0.148]/m_0[F_A]$ as suggested by Smidsrød (195). Figure 3.22 shows the corrected relationships. An increase in acetyl content decreases charge density and electrostatic repulsion and with it the coil volume of the polymer chain.

Figure 3.22: Limiting viscosity number values of the chitosan sample matrix over F_A corrected for differing m_0 in 0.1M HOAc/0.2M NaCl at 25°C



A decrease the polymer coil's hydrodynamic volume will lead to lower limiting viscosity number values.

The implications of a polynomic rather than linear relationship for limiting viscosity number over F_A as can be seen in Figure 3.22 will be elucidated in section 3.4.

3.3.4.2 Dilute solution viscosity and limiting viscosity number values of the CHMS matrix

The limiting viscosity number of CHMS was determined for the solvent system 0.05M NaCl for which a test CHMS material N8 showed linearity of dilute solution viscosity along its dilution range up to a concentration of about 2g/L at 25°C. Beyond the latter dilution the viscosity starts to increase non-linearly due to coil expansion as a result of the polyelectrolyte charge not being compensated at the electrolyte concentration employed and consequent intramolecular electrostatic repulsion. Similar behaviour can be

seen for dilute solution curves of chitosan for dilutions above 0.2g/L. Increasing the electrolyte concentration further in order to obtain linearity for the measuring point of highest concentration was not considered, since this would decrease the overall dilute solution viscosity and along with it the plots slopes and accuracy of this graphical determination. Thus 0.05M NaCl was chosen as a suitable solvent system for dilute solution measurements for CHMS, which is generally less viscous than the corresponding chitosan.

Figure 3.23: Linearity of dilute solution viscosity of CHMS in 0.05M NaCl

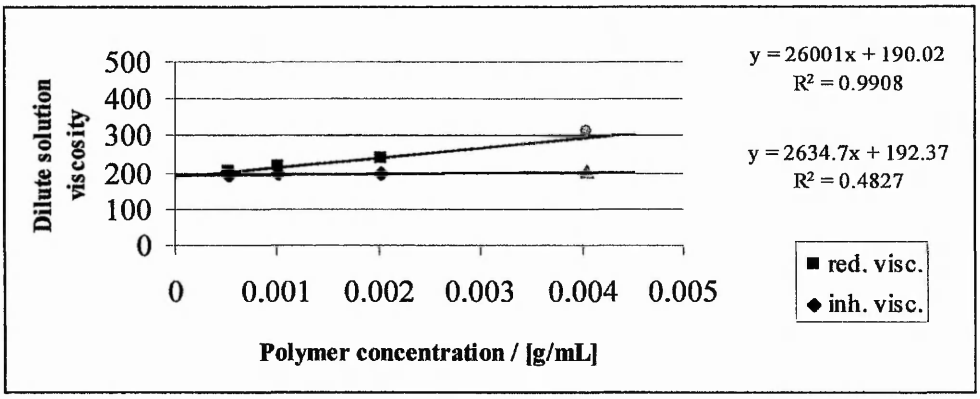
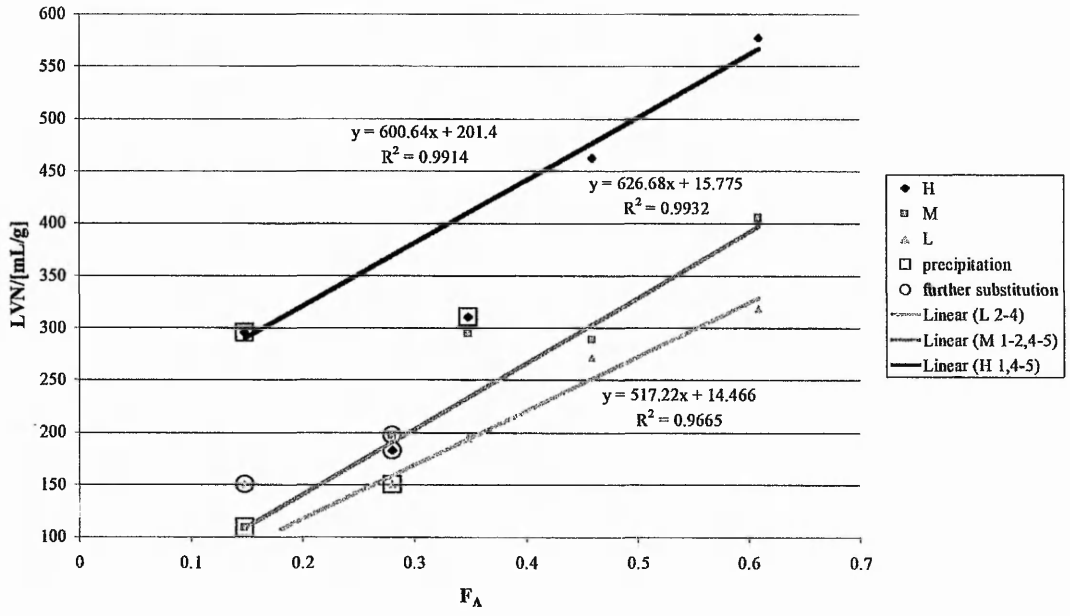


Figure 3.24: Limiting viscosity number values of the CHMS sample matrix over F_A corrected for differing m_0 in 0.05M NaCl at 25°C

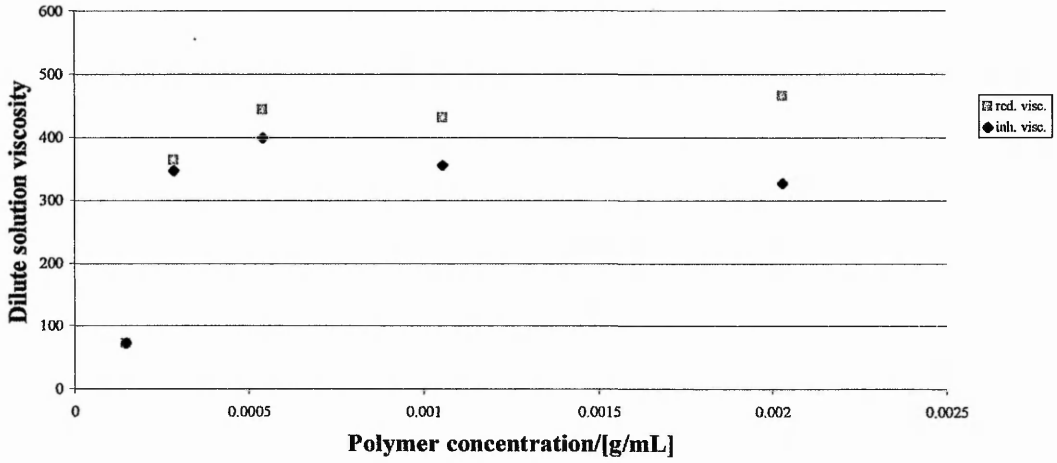


The determined limiting viscosity number values of the CHMS matrix were plotted against the F_A of the initial chitosan taking account of the different m_0 values. A linear regression could be obtained for all three molecular weight fractions omitting obvious escape values. The numbers behind the legend entries for the linear regression plots indicate which data points were employed for generating them. It is further indicated in Figure 3.24 whether the samples were produced by heterogeneous reaction with never-dried chitosan (simple marker), by further substitution (encircled marker) of the latter in cases of non-quantitative reaction or by the dissolution-reprecipitation process (marker in square box). The limiting viscosity number trends do not reflect a relationship to processing conditions, which confirms that reproducible molecular weight distributions were achieved and escape values are not a result of different production methods. Repeated limiting viscosity number measurements of the samples CHMS-H[0.280], CHMS-M[0.280], CHMS-M[0.348] and CHMS-L[0.348] confirmed that the data points are valid.

3.3.5 Dilute solution viscosity of CHMS at low electrolyte concentration

Chitosan as a polyelectrolyte has been shown to deviate from the linear Huggins equation of reduced viscosity *versus* polymer concentration at low electrolyte concentration. Dilution leads to increase in reduced viscosity (156, 194) with progressing decrease in polymer concentration. This increase in reduced viscosity is explained as a dominance of increasing osmotic effects in the immediate environment of the macromolecular coil at low polymer (i.e. also polyelectrolyte) concentration and thus inflation of the polyion coil by solvent molecules. When the decrease of reduced viscosity due to the decrease of intermolecular interaction with decreasing polymer concentration is greater than the increase due to osmotic effects, then the slope again becomes negative (155). At the conditions and ranges regarded in former studies no maximum was observed.
Regarding the reduced and inherent viscosities of CHMS as a negatively charged chitosan derivative in distilled H_2O, typical polyelectrolyte behaviour can be observed.

Figure 3.25: Dilute solution viscosities of CHMS at low electrolyte concentration

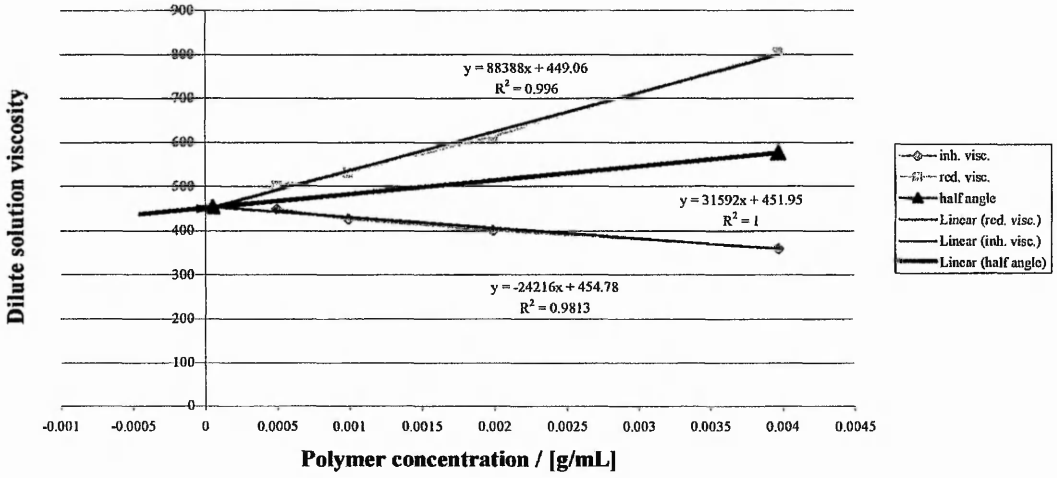


Similar to findings for equally negatively charged hyaluronic acid at low (0.0001M NaCl) electrolyte concentration (236, 237) CHMS with increasing polymer concentration initially shows an increase in dilute solution viscosity due to intermolecular interaction effects being dominant up to a maximum. Beyond the latter the viscosities decrease as a result of osmotic effects gaining importance to eventually increase again as increasing polymer concentration outweighs the osmotic effect. Observing a maximum, rather than increase in viscosity down to very low concentrations is obviously a consequence of very small amounts of residual electrolyte in the employed H_2O. The source may be impurities in the initial H_2O and some decomplexation of the chitosan derivative as taken under consideration for low polymer and electrolyte concentration (see section 3.2.1.4).

3.3.6 Consideration of half angle extrapolation for the improvement of the limiting viscosity number value accuracy

The limiting viscosity number is determined by extrapolation of the inherent or reduced viscosity to its value at zero concentration. While many researchers only employ the intercept of the extension of reduced viscosity and the y-axis the accuracy of the obtained value can be evaluated by considering the regression curves of both dilute viscosities. When plotted on the same graph the inherent viscosity and the reduced viscosity should extrapolate to the same intercept with the y-axis at concentration=0. This can be employed to check the reliability of the measurements (212).

Figure 3.26: Typical example for a diagram of limiting viscosity number determination by viscometry

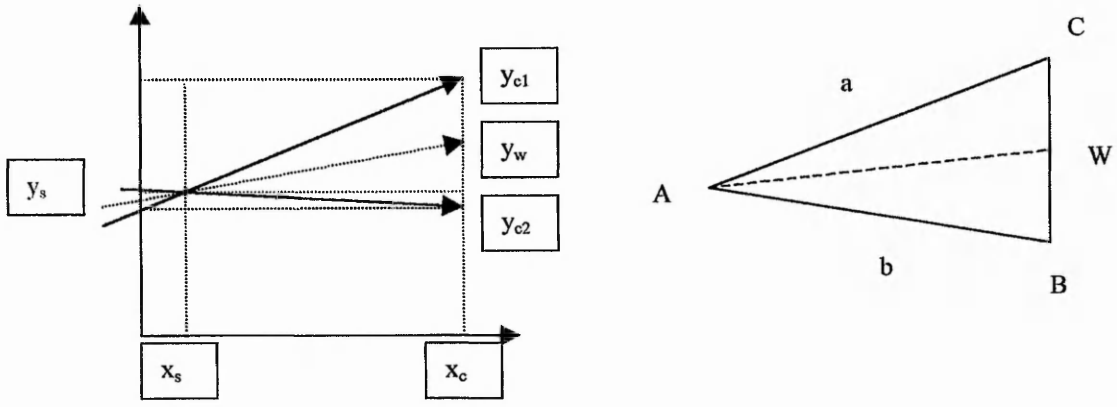


Since it was observed, that in practice the two lines did not meet accurately a method of approximating the true value in between the two intercepts was established. One possibility is presented by taking the mean value of the two. However it was felt that a more accurate approximation theoretically would be obtained by calculating the intercept of the half angle line between the two curves and employing its intercept value with the y-axis. The half angle value would take into account the different slopes of the viscosity curves, which in general are not symmetric to a line drawn parallel to the x-axis, but contribute, in different proportions to the deviation from the true value. In cases where they are symmetric the mean value and a value obtained by half angle extrapolation are equal.

The half angle can be determined by trigonometry and employing the regression curves and their equations calculated in Microsoft® Excel 97 SR-1.

In Figure 3.26 a typical example for a diagram containing plots over 4 concentration dilution points of the reduced viscosity, inherent viscosity and the half angle can be seen. The half angle was calculated by manipulating the regression equations supplied by the software as displayed on above chart. The R-squared values as a measure of congruency between the regression curve and the measured points are given below the respective equation.

Figure 3.27: Schematic diagram of the lines regarded



The intercept of the two curves is calculated from the regression equations:

$y_1=a_1*x_1+b_1$ (reduced viscosity)

$y_2=a_2*x_2+b_2$ (inherent viscosity)

Intercept means, that $y_1=y_2$ and $x_1=x_2$. Thus it follows via arithmetic manipulation that:

$$x_s = \frac{b_2 - b_1}{a_1 - a_2}$$

and

$y_s=a_{1,2}*x_s+b_{1,2}$

x_c is chosen as the concentration of the initial solution and y_{c1} and y_{c2} are calculated from the respective regression curve equations.

The half angle in the triangle ABC or (x_s,y_s) (x_c,y_{c2}) (x_c,y_{c1}) can be obtained from the relationship

$$\overline{BW} : \overline{WC} = b : a$$

This means, that W divides c in a proportion of b:a. It is also known, that

$$c = y_{c1} - y_{c2}$$

The y-value of W can be determined by

$$y_w = y_{c2} + \frac{y_{c1} - y_{c2}}{a+b} * b$$

a and b can be determined by the Pythagorean relationship:

$$a = \sqrt{(x_c - x_s)^2 + (y_{c1} - y_s)^2}$$

and

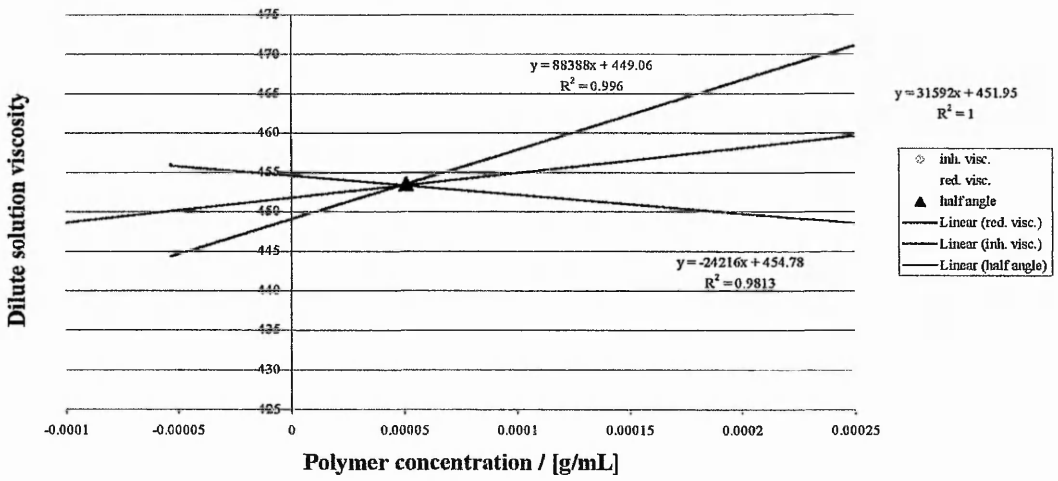
$$b = \sqrt{(x_c - x_s)^2 + (y_s - y_{c2})^2}$$

When x_s, y_s, and x_c, y_w are known, the half angle regression curve can be plotted. The regression equation of the curve yields the intercept with the y-axis which lies between the intercepts obtained from the reduced viscosity plot and the inherent viscosity plot and thus theoretically represents a more accurate value for limiting viscosity number, [η].

As can be seen in Figure 3.28, which is typical for those obtained in the present investigation, the half angle can be calculated sufficiently accurately from regression equations obtained from the software package. However the operator time required for transferring the necessary values as read from the equation in the plot has to be considered. An automatic link of the numbers calculated in the regression equations to further mathematical manipulation is not possible within the software package employed. Since the accuracy in the present study was considerable, and thus the variation of means values from those obtained by half angle extrapolation negligible, the use of the means value

presented values of appreciable precision. The conclusions are that the half angle extrapolation is useful for cases where the deviation of the viscosities' intercepts and the intercept with the y-axis are comparatively large. In the study at hand however, where the experimental precision and accuracy were very high, the use of the mean value between the two points of intercept with the x-axis and the viscosity plots which has been done for chitosan (165), as generally suggested (238), was employed with satisfactory results.

Figure 3.28: Intercept area of Figure 3.27 magnified



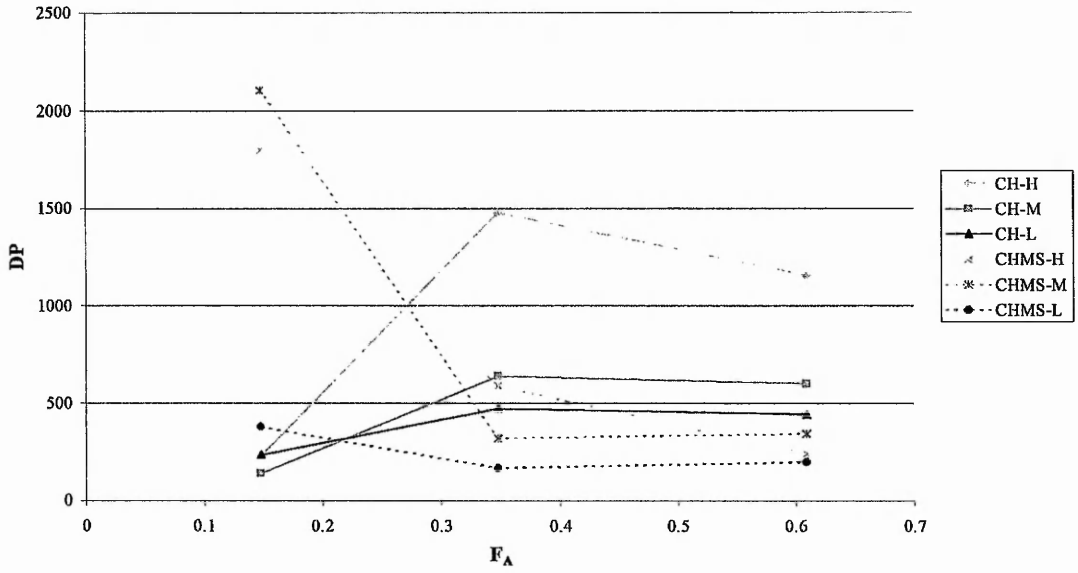
3.3.7 Implications of the limiting viscosity number determined for the two matrices on k and α values for Mark-Houwink equation as well as chain conformation of the materials in solution

3.3.7.1 Molecular weights determined by SEC-MALLS

The molecular weights and polydispersity of the chitosan samplesas well as the corresponding CHMS samples were determined by SEC-MALLS, light scattering being one of few absolute methods of determining molecular weight. Figure 3.29 gives an overview of the degree of polymerisation obtained from molecular weight values. For CHMS a 0.5M buffered solution of FSBS was used in order to prevent decomplexation and thus loss of molecular weight through decrease of m_0 as found in dilute solutions of the chitosan derivative (see section 3.2.1.4). It is advantageous to discuss degree of

polymerisation rather than the actual molecular weight values, since the differences in m_0 due to different F_A are thus eliminated making the values easier to compare.

Figure 3.29: Degree of polymerisation calculated from the determined molecular weight values *versus* F_A for chitosan and corresponding CHMS samples



As can be seen in Figure 3.29 the values of DP are constant with good approximation for the medium and low molecular weight fractions for $F_A \geq 0.348$ for chitosan and the converted chitosan, confirming the non-degradative nature of the conversion process. The high molecular weight fraction in the same range of F_A shows a parallel decrease in molecular weight from F_A 0.348 to 0.609. The values for chitosan are seemingly too high, since only the MW fraction $\geq 4.00*10^5$ da was considered due to technical issues, thus cutting off the low molecular weight contribution to the average molecular weight. In the F_A range of high amine content and thus high polycationic character of the polymer the chitosan shows a seeming decrease in average molecular weight to nearly the same value of degree of polymerisation for the high molecular weight as well as medium and low molecular weight fractions. The most likely cause for this finding is adsorption of those samples having the most cationic character onto the column and especially their high molecular weight fractions. In the same range of high polyelectrolyte character the converted anionic chitosan derivative shows a seeming increase in DP however not congruent with their qualitative molecular weight specification CHMS-H, -M, -L. This

effect is somewhat more difficult to explain, but might be a consequence of steric inter-chain interaction i.e. "entanglement" of the chain due to the more lengthy subsituents and the high concentration of them. The entanglement would lead to chain agglomerates going into solution thus emulating single molecules with seemingly to high molecular weight. The consequences for the actual DP of CHMS and thus the corresponding chitosan are that the values for the medium and low molecular fraction are obtained by averaging the values of DP for $F_A \geq 0.348$. For the high molecular weight fraction the value at F_A=0.348 is employed. This is justified by the parallelity of the DP values for the medium and low molecular weight fractions in the regarded F_A range as well as the parallel decrease of average DP for the high molecular weight fraction of chitosan and the corresponding CHMS material indicating that indeed at some stage of the preparation of CH-H[0.609] degradation had occurred. From these DP values of CHMS the corresponding weight average molecular weights can be back calculated for the determination Mark-Houwink relationships for CHMS and the initial chitosan.

Table 3.22: DP values determined from weight average molecular weights from SEC-MALLS

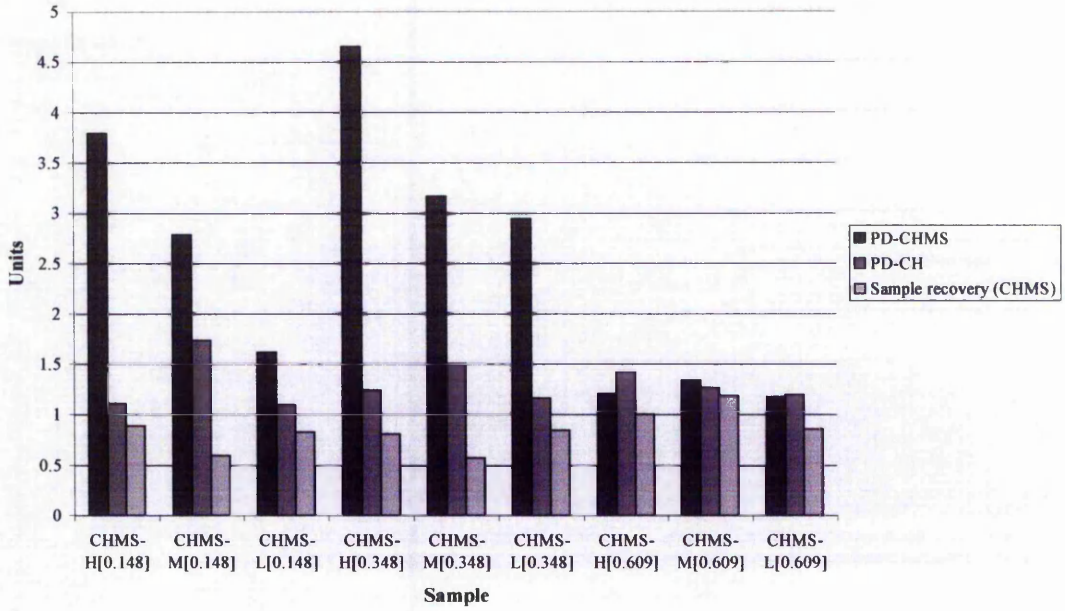
	DP values calculated from weight average MW/m_0		
	CHMS-H	**CHMS-M**	**CHMS-L**
0.148	1798	2106	379
0.348	591	322	168
0.609	244	346	201
Average DP	**591**	**334**	**184**

3.3.7.2 k and α relationships of molecular weight for Mark-Houwink equation

The Mark-Houwink relationship is only rigorously valid when the limiting viscosity number corresponds to a monodisperse material (155, 237). Thus a low value for polydispersity was desirable. Figure 3.30 shows that the polydispersity of CHMS decreases with increasing random chain degradation with 2 being the value for a Gaussian random distribution. The comparatively low and constant values of chitosan are a result of only the molecular weight fraction above 40,000 da being considered as a result of technical issues.

The chromatograms (shape of detector signal over time) of CHMS (see Appendix) confirmed the presence of a molecular weight continuum without any peculiarities.

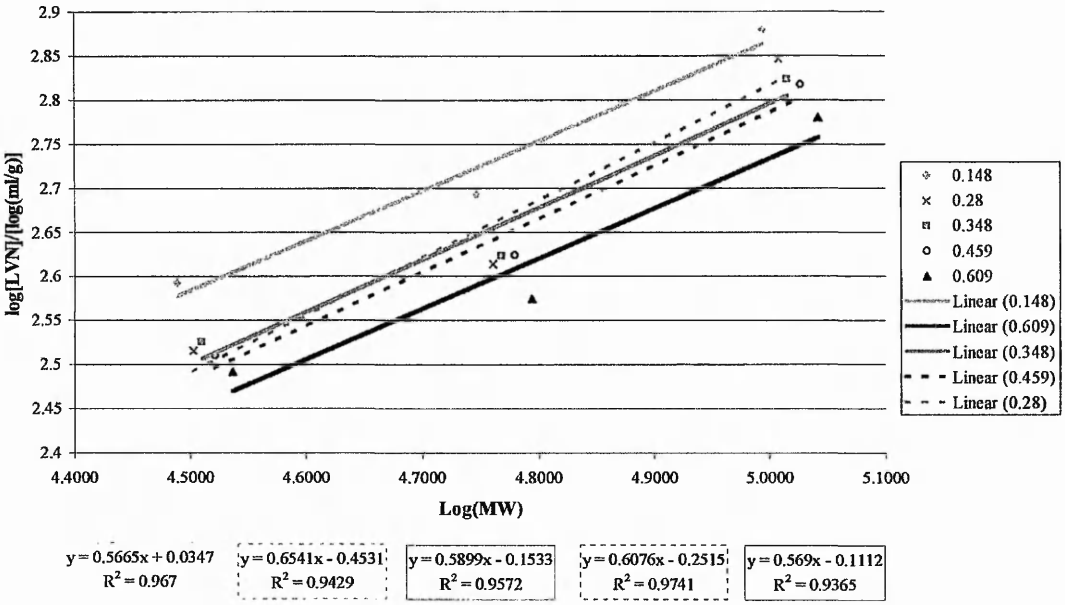
Figure 3.30: Polydispersities (Mw/Mn) of CHMS and initial chitosan samples and sample recovery by SEC-MALLS



Plotting the logarithms of the experimentally obtained limiting viscosity numbers as a function of the logarithms of molecular weight produces as linear regression curve with the slope representing the α value and the intercept with the y-axis equating to the logarithm of k in a double logarithmic plot of the Mark-Houwink equation.

Figure 3.31 shows the obtained functions for chitosans[0.148], [0.280], [0.348], [0.459] and [0.609]. The slope and thus α value for chitosan[0.280] is comparatively high leading to a crossover with the curve for chitosan[0.348] within the molecular weight range regarded.

Figure 3.31: Determination of Mark-Houwink parameters for chitosan from LVN as measured



The determined discreet k and α parameters for chitosan at five different F_A fractions and weight average molecular weights in the range of $3.08 * 10^4$ to $1.10 * 10^5$ da are summarised in Table 3.23.

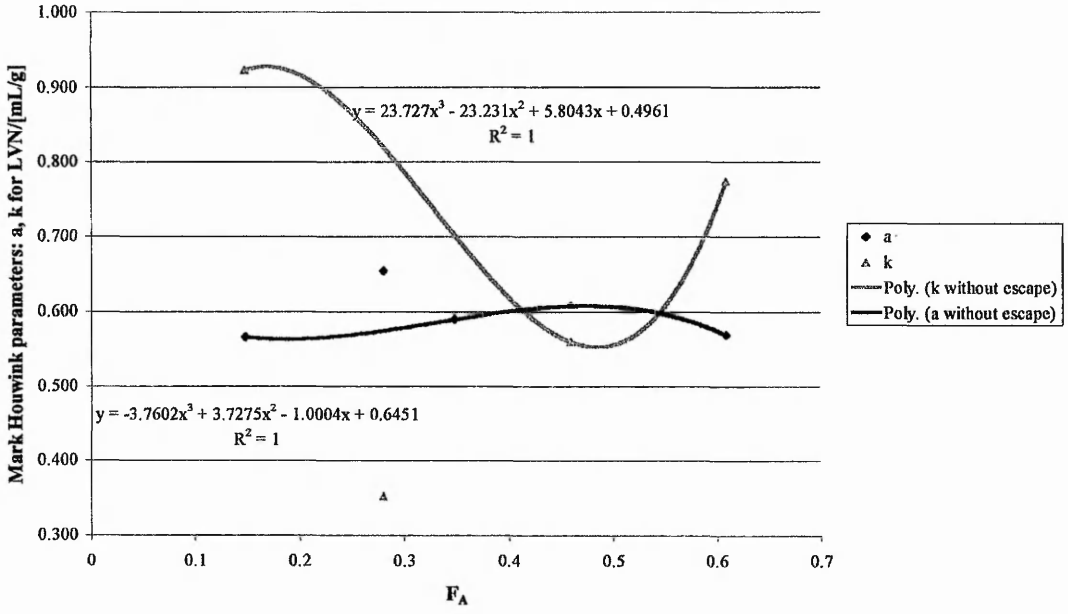
Table 3.23 Mark-Houwink parameters for chitosan

F_A	k*	α
0.148	0.923209	0.567
0.280	0.35229	0.654
0.348	0.702587	0.590
0.459	0.560402	0.608
0.609	0.774105	0.569

* valid for limiting viscosity number/[mL/g]

Figure 3.32 gives a graphic depiction of the change of k and α values *versus* F_A. The relationship for F_A=0.280 seems to be an escape. Omitting the latter, k and α can be expressed as 3rd order polynoms for the regarded range $0.148 \leq FA \geq 0.609$. The conformational implications of k and α values will be discussed in section 3.3.6.3.

Figure 3.32 Mark-Houwink parameters of chitosan as functions of F_A



From these results the Mark-Houwink relationships predicted for chitosan can be presented as a function of F_A in the following way

$$LVN(F_A) = k(F_A) * MW^{\alpha(F_A)}$$

with f and g being functions of F_A

$$k(F_A) = 23.727 * F_A^{\ 3} + 23.231 * F_A^{\ 2} - 5.8043 * F_A + 0.4961$$

$$\alpha(F_A) = -3.7602 * F_A^{\ 3} + 3.7275 * F_A^{\ 2} - 1.004 * F_A + 0.6451$$

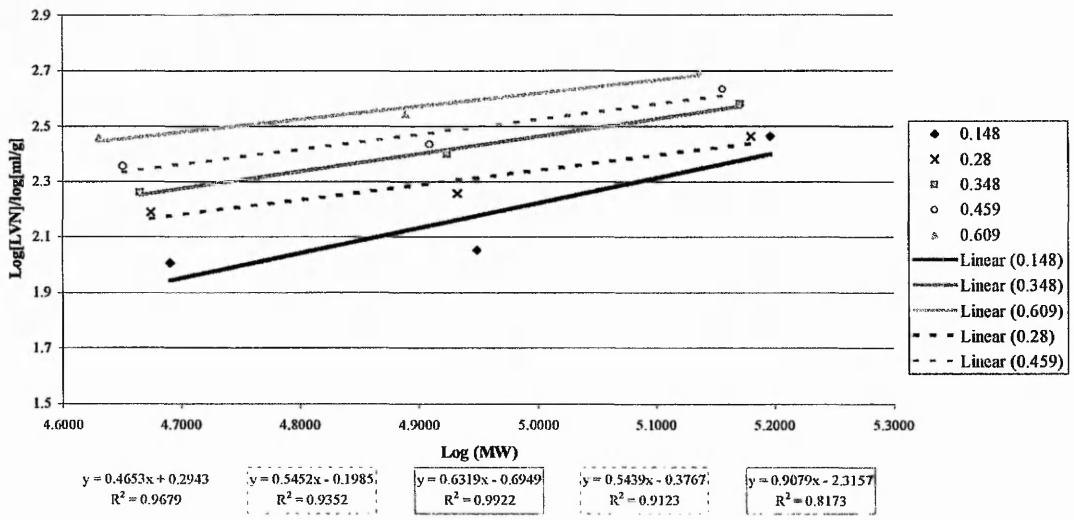
The Mark-Houwink relationships for CHMS were determined in the same way. However instead of employing the actually measured limiting viscosity number values it was felt that due to the higher variation of values (see Figure 3.24) the use of theoretical values as back calculated from the regression equations was appropriate.
The regression equations of Limiting viscosity number *versus* F_A as measured (not corrected for different m_0) are as follows:

CHMS-H: $LVN/[g/mL] = 444.91*F_A+226.52$

CHMS-M: $LVN/[g/mL] = 515.02*F_A+36.477$

CHMS-L: $LVN/[g/mL] = 407.99*F_A+40.605$

Figure 3.33: Determination of Mark-Houwink parameters for CHMS from LVN as calculated from regression curves



In Figure 3.33 it can be seen that the double logarithmic plots for CHMS follow a clear trend of increasing slope, that is α, with decreasing x-axis intercept, that is log(k), with increase in F_A apart from the curve for CHMS[0.280]. Table 3.24 gives the discreet k and α values for CHMS with 5 data points in the F_A range of 0.148 to 0.609 and a weight average molecular weight range of 4.28 $*10^4$ to 1.57 $*10^5$ da.

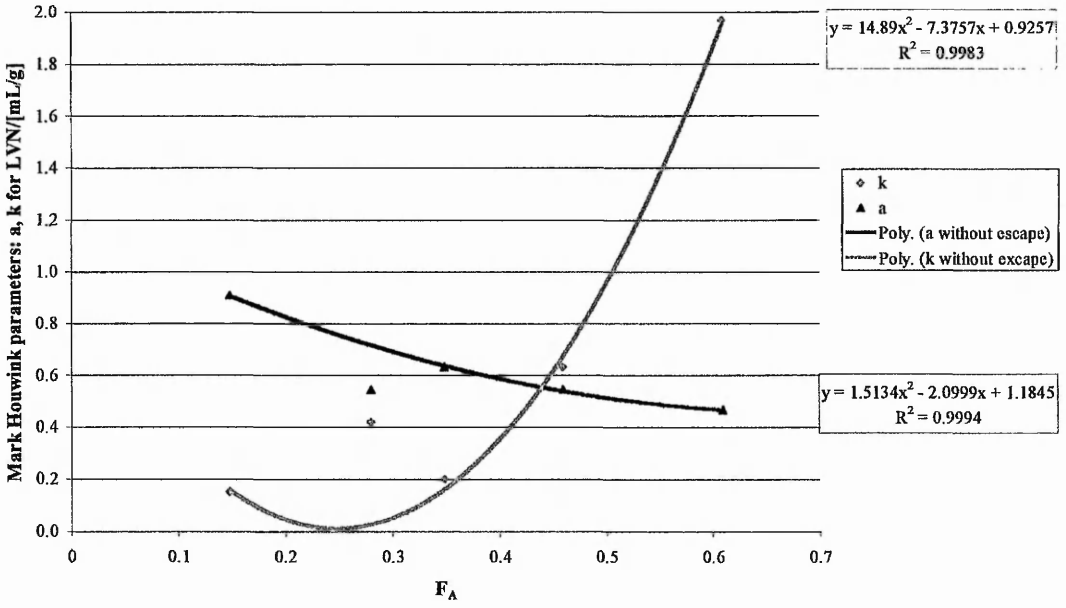
Table 3.24 Mark-Houwink parameters for CHMS

F_A	k*	α
0.148	0.152	0.908
0.280	0.420	0.544
0.348	0.202	0.632
0.459	0.633	0.545
0.609	1.969	0.465

* valid for limiting viscosity number/[mL/g]

A graphic depiction of the k and α values of CHMS is presented in Figure 3.34. Here it becomes even more apparent, that the value for F_A=[0.280] is an escape value. The latter was omitted for generating a regression function for the change of the parameters with F_A as has been done for the equivalent relationships for chitosan as can be seen in Figure 3.32. The conformational implications of these relationships are discussed in section 3.3.6.3. The regression curves with best fit are polynomial functions of 2nd order with k exhibiting a minimum of value 0.012 at F_A=0.248.

Figure 3.34 Mark-Houwink parameters of CHMS as functions of F_A



Resulting possible predictions for Mark-Houwink relationships for CHMS can be presented as a function of F_A in the following way

$$LVN(F_A) = k(F_A) * MW^{\alpha(F_A)}$$

with k and α being functions of F_A

$$k(F_A) = 14.89F_A^{\,2} - 7.3757 * F_A + 0.9257$$

$$\alpha(F_A) = 1.5134 * F_A^{\,2} - 2.0999 * F_A + 1.1845$$

Since k and α values are related to the conformation of the polymer in solution they are specific to the solvent system and temperature employed for their determination. Thus it is not really feasible to directly compare reported values and relationships unless they have been determined in the same solvent system and hence at the same ionic strength. However relationships for limiting viscosity numbers of varying chitosan samples in different solvent systems have been found linear (158) for moderately concentrated electrolyte compositions of acetic acid and sodium acetate buffer or sodium chloride at slightly varying temperatures. This suggests that trends in similar solvent systems can still be expected to be similar albeit at differing absolute values.

3.3.7.3 Consequences for chain conformation of chitosan and CHMS in dilute solution

a) General

Limiting viscosity number is a measure of hydrodynamic volume and subsequently chain conformation of a polymer at a certain molecular weight. The conformation, as in size and shape, of a polymer in dilute solution is dependent on intra-molecular forces such as charge repulsion in the case of polyelectrolytes like chitosan. It also depends on the stiffness of the polymer backbone as related to rotational freedom around the glycosidic linkage for linear polysaccharides such as chitosan. Comparing chitosans of F_A 0.11 and 0.42 a more flexible molecule with however more extended shape was found for the first more highly charged material (162). From the dilute solution measurements carried out, k and α values from the Mark-Houwink equation and K_H, the Huggins parameter determined from the slope of reduced viscosity against polymer solutions can be extracted. These parameters give an approximate idea of conformational situations of polymers in solution. For example the K_H can approach values of up to 2 for solid uncharged spherical particles with a decreasing trend for more extended shapes, while more flexible biomolecules exhibit values around 0.35. Mark-Houwink parameter k is related to polymer conformation however in a more complex way than α. For the conformational extremes compact sphere, rigid rod and random coil α values have been reported in the order 0, 1.8 and 0.5-0.8 respectively (237) or similarly 0.65 and 0.85 in good solvents for soft coils of molecules. Higher values indicate stiffness and molecular asymmetry. Over some 4 powers of molecular weight a is constant, but at higher molecular weight a will decrease, since the

solvent does not drain as freely and at lower molecular weight a is predicted to increase (155).

b) Chitosan

Figure 3.35a: LVN of CH *versus* F_A*

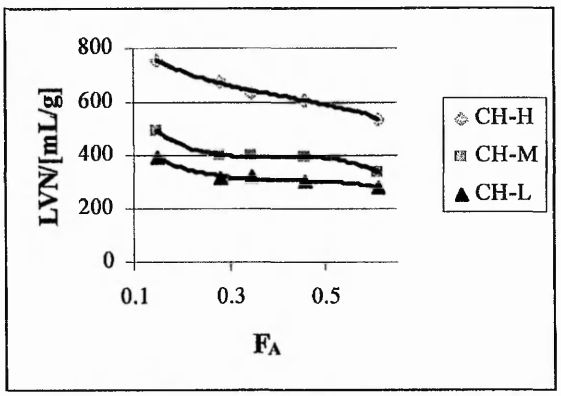


Figure 3.35b: k, α of CH *versus* F_A

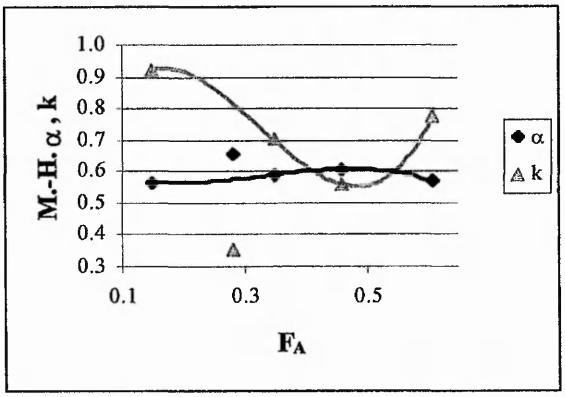
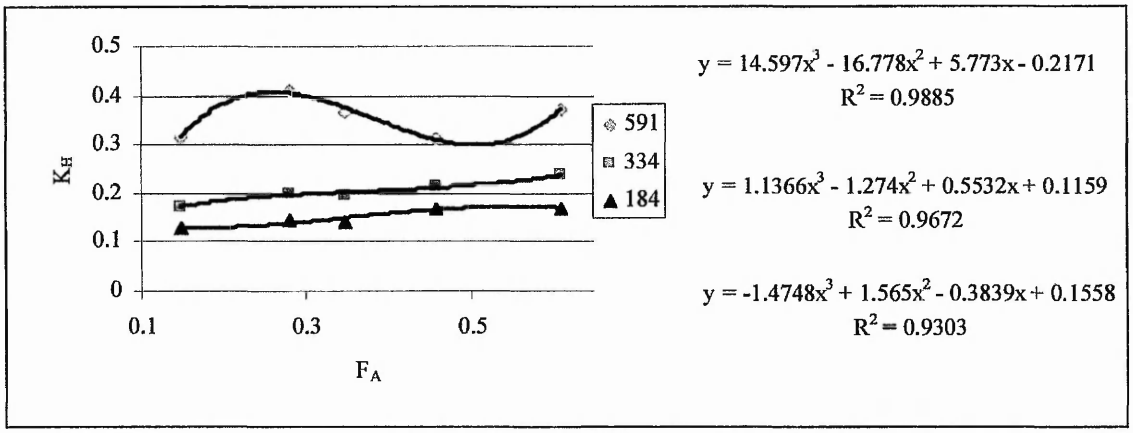


Figure 3.35c: Huggins parameter K_H* *versus* F_A for chitosans with three different DP



In Figure 3.35b (for more detail and regression functions see Figure 3.32) the k and α values are plotted against F_A. Consideration of the data points suggests a 3rd order relationship for both values. The α values are in the overall range of 0.567-0.608 and thus well in the region of random coils. The change of α with increasing F_A is initially very flat up to a F_A value of about 0.25. The stiffness parameter *B* for chitosan has been reported

* Corrected for m_0

near constant for F_A up to 0.15 (165) and little change was claimed up to a F_A value of about 0.28 (239). In accordance the intrinsic persistance length as a measure of chain stiffness was found constant for values up to F_A=0.25* and steadily increasing with higher *N*-acetyl concentrations (163). The above findings for α and the reports on *B* and intrinsic persistence length suggest a charge density controlled conformation up to $F_A \cong 0.25$. Looking at the decrease of LVN *versus* F_A in this range in Figure 3.35a (see also Figure 3.22 for equations and more detail), it can be seen that the hydrodynamic volume is decreasing as a result of decreasing electrostatic repulsion. The trends for Huggins constants as a ballpark indicator for conformation can be seen in Figure 3.35c for the three chitosans with different DP. All three, as suggested functions of 3rd order, show an extreme value at around 0.25. Their absolute values are around 0.35 as expected for the comparatively flexible polysaccharides. The most pronounced change is shown in the order from high molecular weight chitosan to lower molecular weights. This can be explained by the higher statistical number of general conformations a longer chain can assume as opposed to shorter chains, thus making changes of conformational characteristics show up more clearly. Wales-Van Holde ratios as conformational indices have been found to vary strongly for chitosans produced by nitrous acid degradation similar to the chitosans discussed here. Thus a different conformation of the two different molecular weight materials was suggested (162). This might also explain a change of initial curvature from strongly convex (=decrease of slope# *versus* F_A) for the high molecular weight material to less pronouncedly convex for the medium molecular weight material and slightly concave (=decrease in slope#) for the low molecular weight material. A deviation from assumed free drainage for larger molecules (155) may be another cause for findings of different LVN trends.

Beyond $F_A \cong 0.25$, the α value increases up to a maximum at about $F_A \cong 0.5$ to reach its initial value at about 0.61. The three stages from low (0.25) to high *N*-acetylation (0.61) via moderate values (0.5) could be seen as corresponding to an initially charge repulsion dominant coil conformation, which then becomes stiffer, when increasing *N*-acetyl group concentration increases the probability for intra-chain hydrogen bonding and thus the

* This was found for heterogenously produced chitosans, while for homogeneously produced chitosans the intrinsic persistance length was reported to inrease moderately with increase in F_A. In the chitosans present in this study there is a heterogeneous contribution to F_A of 0.148.

Slope = first derivative

generation of stiffer segments (see illustrated in Figure 1.3) as predicted in molecular modelling for intrinsic persistence length (163). At lower random F_A the probability of having several stiffening sequences is low, while the still fairly flexible chain can accommodate small amounts of stiff elements without much change. At higher F_A the stiffness gains more influence on the molecular expansion counteracting the influence of loss of repulsion due to loss of charge density.

The trend of the α values, while overall well within random coil range gain more rod like contribution (increase towards 1.8 for ideal rod shape) from 0.25 to 0.5, after which the α values for samples approaching F_A 0.61 show a trend towards lower values for increasing compacting of coil conformation. The latter might be due to non-polar intra-chain interaction of comparatively stiff *N*-acetyl glucosamine of increasing sequence length. The coil shape changes could accommodate the well-known and here confirmed tendency of decreasing LVN for increasing *N*-acetyl concentration, despite the latter's stiffening consequences, which otherwise would result in coil extension and LVN increase. The trends away from and towards spherical coil shape are also reflected in the 3rd order approximation of determined Huggins constants for $0.25 \leq F_A \geq 0.61$. Their curvature alternates in the same area as changes for the trends of α are apparent, thus suggesting less spherical contribution for values differing more from 2 and again more spherical proportions for values moving towards 2. The LVN values show a similar trend for alternating curvature, confirming the findings for K_H and α

c) CHMS derivative of chitosan

Most striking in the comparison of the two polyelectrolyte is that an increase in F_A leads to an overall decrease in limiting viscosity number for chitosan as found previously (160), while it clearly increases the values for the CHMS polyion. Secondly the CHMS derivative of chitosan is clearly overall less extended than its parent material, as can be qualitatively clearly seen by comparing the absolute LVN values and trends (Figures 3.22 and 3.24). At a much lower electrolyte concentration of 0.05M NaCl, compared to chitosan at 0.1M HOAc/0.2M NaCl, the CHMS at high charge density shows less than half the hydrodynamic volume of the corresponding chitosan. While the amine concentration in chitosan increases the coil dimensions due to charge repulsion, the sodium *N*-methylsulphonate group's charge is further removed from the polysaccharide backbone by a carbon, a sulphur and oxygen linkage (see Figure 3.36b) compared to the amine group's

charge, which is in close proximity to the carbon atom nearest the chain flexibility determining glycosidic linkage (compare Figure 3.36a). The spacing effect of the former group between polymer backbone/glycosidic linkage may decrease the rotational hindrance due to electrostatic repulsion, as the charges are further removed from each other on rotation. The conformational freedom of charge location introduced allows charge repulsion without compromising the rotational freedom around the glycosidic bond. However these "outreaching" charged appendages may inflict longer-range electrostatic effects with other parts of the molecular chain. The increase of linkages with rotational freedom between the polymer backbone and the charge carrying

Figure 3.36a: Structure of chitosan in solution

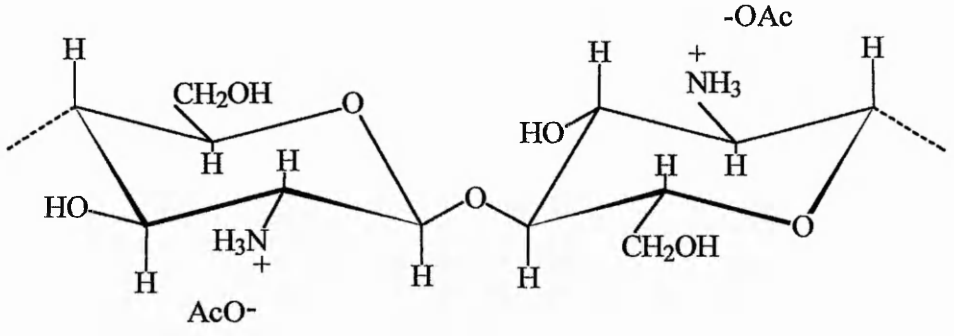
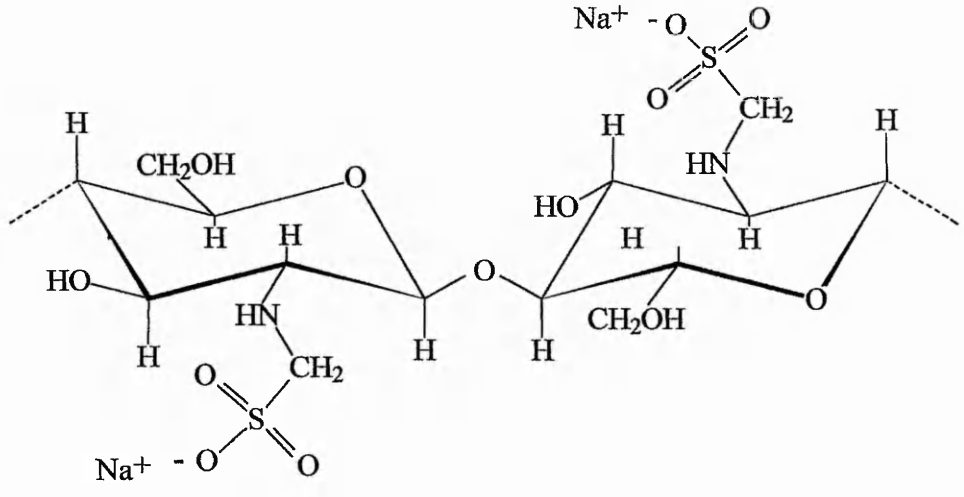


Figure 3.3b: Structure of CHMS



atom increases the statistical number of possible locations for the charge and thus gives more scope for possible orientation of the molecule chain. The effect of lower hydrodynamic volume with lower acetyl group concentration is coupled with the thus decreasing intra-hydrogen bonding and the latter's chain conformation defining potential. The combination of comparatively high polymer coil volume at comparatively low charge density may account for findings in metachromatic titration. Here the interaction was found to be decreasingly pronounced for increasing F_A values in CHMS possible due to decreasing intra-coil osmotic activity.

Looking at the rough trends for conformational indices α and K_H against F_A in Figures 3.37a (for more detail and equations see Figure 3.34) and 3.37b gives an idea of change of polymer coil shape with change in chemical composition. For Figure 3.37b the values of K_H determined in the same course as limiting viscosity number values that were not

Figure 3.37a: k, α of CHMS *versus* F_A

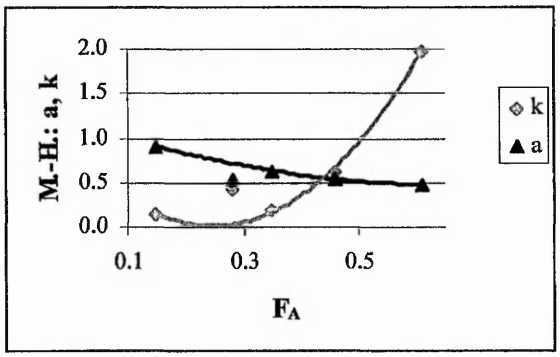
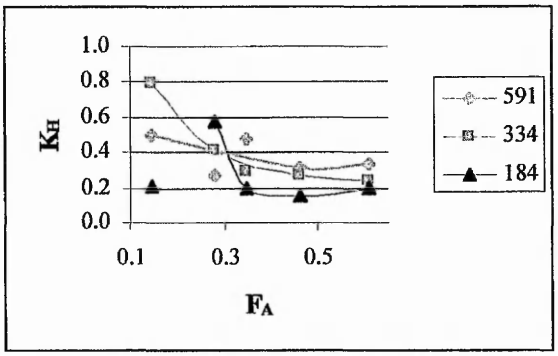


Figure 3.37b: Huggins parameter K_H *versus* F_A for CHMS with different DP values



considered for the relationships of LVN against F_A for CHMS (see also Figure 3.24) were omitted for obtaining the trends for Huggins constant in dependence of F_A. The graphic depiction of tendencies was carried out by smooth line interpolation between the considered data points. Although the trends thus obtained for the materials of high and medium DP (591, 334) can be approximated with equations of 3rd order with very good visual aggreement, the same procedure was not justified for the position of data points for the low DP (184) material. Hence regression equations were not considered justified at this point, however the graphic tendencies provide qualitative conformational information. In

agreement and confirming the conclusions drawn above for the findings for limiting viscosity number and the electrostatic differences between chitosan and CHMS the trends for α and K_H allow the following predictions. Starting off with the highly de-*N*-acetylated material and thus materials with a high density of charged molecules the α value of 0.91, which is outside the range for random coil toward the values for rigid rod steadily decreases with F_A and reaches the random coil band for α at $F_A \cong 0.2$. This suggests with the findings of low hydrodynamic volume at low F_A that at high density of the mid length charged substituents a more rod like conformation is assumed to accommodate electrostatic repulsion rather than a more extended random coil or sphere. The rough trends observed in Figure 3.36b for Huggins constants equally suggest a more rigid conformation for low F_A CHMS material and more flexibility for decreasing content of the charged substituent.

3.3.8 Summary

- A matrix of chitosan materials was prepared from one sample of deacetylated commercial high DP chitosan with three discreet molecular weight distribution continua in the qualitative order high, medium and low by homogeneous random chain scission with nitrous acid. Reproducible homogenous re-*N*-acetylation yielded chitosans with 5 discreet values of re-*N*-acetylation for each molecular weight fraction. The F_A covered a wide range from 0.148 to 0.609
- The random character of chain scission was confirmed by SEC-MALLS polydispersity findings of approaching the value 2 with progressing chain degradation. The latter polydispersity is the value for random molecular weight distribution
- The reproducibility of each F_A value across the three different molecular weight materials was established by dye adsorption analysis
- It could be shown by SEC-MALLS that the DP during the here employed process of reacetylation was largely retained as previously suggested from capillary viscometry and ultracentrifugation (195)
- A corresponding matrix of CHMS materials only differing from their parent chitosan materials in possessing Sodium *N*-methylsulphonate groups instead of the amine group was prepared by quantitative Sodium *N*-methylsulphonation of the matrix chitosan's amine groups
- Complete reaction of amine groups in chitosan to CHMS was monitored by solubility tests and confirmed by metachromatic titration
- The non-degradative nature of the solution process and conversion of chitosan to CHMS was shown from DP retention data obtained by SEC-MALLS
- The limiting viscosity number values of the 15 samples of each, the chitosan as well as the CHMS matrix (30 samples overall) were determined by capillary viscometry
- A solvent system suitably salting out the polyelectrolyte character of CHMS to obtain linear plots for dilute solution viscosity *versus* polymer concentration was found in 0.05M NaCl solution
- CHMS at low salt concentration showed a maximum of dilute solution viscosities similar to that found for e.g. hyaluronic acid (236)
- The decrease of Limiting viscosity number *versus* increasing F_A for chitosan could be approximated with 3rd order equations with excellent agreement

- The trend of Limiting viscosity number *versus* F_A for CHMS was approximated as linear and of overall inverse relationship compared to chitosan, namely the increase in F_A lead to an increase in hydrodynamic volume
- The average degree of polymerisation of the CHMS samples was determined from weight average molecular weight determined from SEC-MALLS
- Applying the obtained DP values for chitosan and CHMS Mark-Houwink relationships for chitosans in the weight average molecular weight of $3.08*10^4$ to $1.10*10^5$ da and a F_A range of 0.148 to 0.609 were calculated
- The k and α parameters in dependence of F_A were approximated as 3rd order functions fitting the experimentally determined data points
- Employing the experimentally determined k and α values for CHMS against F_A binomial equations with excellent agreement could be found
- Looking at the findings for Limiting viscosity number, α-value and Huggins *versus* F_A constant 3 stages in conformational change in chitosan in dilute solution could be proposed, up to 0.25, up to 0.5 and beyond.

4. Conclusions

New methods for determining molecular weights

The molecular weight of chitosan can be determined by a novel method. In a non-depolymerising solubilising process chitosan can be converted into an anionic polymer with the same degree of polymerisation as the initial material. The advantage is that previously encountered adsorption of the cationic chitosan onto an anionic SEC column is eliminated, giving greater sample recovery and thus more realistic and complete molecular weight distributions coming off the column for subsequent detection by e.g. MALLS. From the thus more accurate absolute degrees of polymerisation obtained for the converted material CHMS and hence for the initial chitosan more accurate Mark-Houwink relationships for either material can be determined as a convenient low cost method of estimating their molecular weight. The more accurate Mark-Houwink parameters k and α give more realistic indices for the dilute solution conformation of chitosan and the first conformational information on CHMS.

Conformation

The findings for dilute solution viscosities show that three conformational changes in the range of *N*-acetylation from $\cong$0.0 up to 0.61 for chitosan can be identified. From the determined α values, and confirmed by Huggins constants and trend of limiting viscosity number *versus* F_A, the following mechanisms are proposed.

The conformational changes are controlled by electrostatic effects up to $F_A \cong 0.25$, a range in which the comparatively high flexibility is nearly constant*, however the coil is extended resulting in a high hydrodynamic volume and limiting viscosity number.

For the compositional range $0.25 \leq F_A \geq 0.5$ (approximately) the increasing number of *N*-acetyl groups, and thus the increasing probability of longer sequences of chain links with low rotational freedom, gain importance and with it chain stiffening and coil deforming become effective. There is a counteraction of the coil expanding force of electrostatic repulsion against coil compacting resulting in a change of shape towards a more rod shaped particle, as confirmed by findings for limiting viscosity number values. The latter's

* At low substituent concentration the probability of finding neighbouring *N*-acetyl groups is fairly low, so that the stiffening effect from singular hydrogen bonds can be accommodated in the highly flexible chain without significant increase in stiffness.

response shows a trend of decreasing hydrodynamic volume with a large plateau in the regarded range.
For the compositional range $0.5 \leq F_A \geq 0.61$ (end of regarded range) the increasing numbers and sequences of hydrophobic stiff chain links provide scope for intra-molecular interaction aided by the decrease in electrostatic repulsion. This leads to more compact conformations as reflected in smaller hydrodynamic volumes.

The equally ionic CHMS materials, despite their corresponding nominal charge density per monomeric unit, were found to exhibit dilute solution behaviour and thus conformational states differing strongly from that of the initial chitosans. This is explained by the spacing effect of the sodium *N*-methylsulphonate group, which removes the charge from the vicinity of the glycosidic linkage thus improving rotational freedom of the monomer units as compared to chitosan. Hence CHMS is generally less extended than chitosan as reflected in lower limiting viscosity number values. The coil shape changes from a conformation with comparatively high rod-like contribution to reach the area of random coil with F_A increasing from 0.148 to $\cong$0.2. The longer substituents seem to pose an obstruction to redissolution of highly sodium *N*-methylsulphonated materials, possibly chain entanglements arising from the more branched nature of the polymer chain, leading to pseudo solutions with solvated polymer aggregates. This phenomenon and its implications on SEC-MALLS may need to be revisited.

Routes for preparation of membranes and other formed-in-place entities
In keeping with findings for cellulose the organosolubility of the derivatised chitosan increased with an increase in degree of overall acylation. Novel chitosan derivatives as shown for di-*O*-butyryl-*N*-hexanoylchitosan as a first of many possible homologues of the reported di-*O*-butyrylchitin can be produced and solubilised in simple organic solvents like acetone and MeOH. This provides scope for the preparation of membrane materials with their pore sizes tailor-made for different separation and transport processes by targeted and selective substitution. In order to make the potential materials feasible for industrial application however it would be desirable to find ways of preparation with lower reagent and processing requirements.
As a second route to chitosan-based membranes with specific pore sizes, chitosan can be crosslinked with environmentally and potentially biomedically viable agents like citric acid

to protect the material from dissolution in acidic media and in order to control the pore size by degree of crosslinking.
Since the reactant is added to the solution before shaping the desired form, such as films in the present study, the method has got further potential for insolubilising other forms like e.g. fibres, as long as the required curing step can be realised.

Dye interaction as an absolute technique with a wide scope of application

It has been shown that dye interaction methods such as dye adsorption and metachromatic titration for determination of equivalent weight of positively charged substitutents* has got a much larger scope than being an absolute technique for the analysis of chemical composition in chitin/chitosan copolymer. From the principle employed for relating the equivalent weight to the amine content in the chitin/chitosan continuum, similar relationships for any complex system of chitosan derivatives with initial *N*-acetyl group as primary substituent and non-protonatable and protonatable higher substituents in any proportion can be derived. As a precondition the degree of substitution of a material must be established before introducing each new substituent. However the scope of this method extends beyond linear systems to networked polymers. The latter's degree of crosslinking as compared for different materials can be estimated by this method, which is particularly interesting for established techniques like controlling pore sizes of membranes by crosslinking.

***N*-acetylation**

The efficiency of *N*-acetylation can be improved by an optimum addition of co-solvent ether to the aqueous acetic acid/MeOH solvent system. The reaction efficiency of acetic anhydride can be approximated with a 2nd order equation at high confidence levels. This is very useful for possible large-scale applications of homogeneously reacetylated chitosans in e.g. selective and controled substitution in the biomedical field and general separation processes. The required anhydride amounts for the respective desired F_A values can be predicted.

* Positively charged under the conditions employed during analysis

Miscellaneous

The syneresis behaviour of Hirano chitin gels can be influenced by parameters such as F_A, molecular weight and co-solvent proportions.

A to date unpublished *N*-carbamyl (-NH-CO-NH_2) derivative of chitosan structurally very similar to *N*-acetyl chitosan (-NH-CO-CH_3) can be prepared as materials with similar solubility behaviour to that of chitosan/chitin copolymer, forming clear solutions up to an overall degree of *N*-substitution of 0.62 and cloudy solutions up to 0.75. The gels obtained in the reaction can be mechanically disintegrated much more easily than Hirano chitin gels.

More accurate values of intrinsic viscosity by capillary viscometry can be extrapolated by half angle considerations. However a more accurate software package than the one at hand needs to be employed for the determination of regression equations for the accuracy required here.

Further Research

Leading out of this work the following immediate issues are in brief proposed for further investigation.

Consistent deviation of values for materials of F_A =0.28 has been treated as an escape value. However, since the value is in the general area of suggested conformational change more data is required to exclude more complex and non-random reasons for this finding.

Plans are in place to evaluate the k and α relationships established here for homogeneously re-*N*-acetylated chitosans of similar molecular weight distribution characteristics for materials with molecular weight distributions and acetylation patterns differing from the materials employed in this study. Determination of the stiffness parameter *B* for the novel CHMS derivatives in comparison with the initial chitosans to further the conformational understanding of both polymers is hoped to be realised in the near future.

The different routes for the preparation of membranes and other forms like fibres, beads, sponges, etc. are promising and no doubt their process requirements can be optimised in foreseeable time. In particular the formation of homogeneous blend of CHMS and anionic polyelectrolytes, and the subsequent reconstruction of chitosan, opens up a wide field of combinatorial possibilities. With the wide range of natural and synthetic polyanions available and mixing continua imaginable as well as the possibility of further linear and network functionalisation of recovered chitosan at varying degrees of initial F_A, the opportunities for creating materials with desired properties would appear to be limitless.

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Appendix

Publications and dissertations

Published Papers

- *Syneresis aspects of chitosan based gel systems* in: Advan. Chitin Sci., Vol.4, M. G. Peter, A. Domard and R. A. A. Muzzarelli, Eds. University of Potsdam, 2000. ISBN 3-9806494-5-8

- *Aspects of chitosan-dye interactions: flocculation and metachromasy*, Presented at: 6th European Training Course on Carbohydrates, Carbohydrate Research Foundation, The Hague, The Netherlands, held at Lajos Kossuth University, Debrecen, Hungary, 2000

Papers in preparation (working titles):

- *Determination of derivation patterns in chitosan/chitin co-polymer derivatives with protonable and non-protonable secondary substituents by dye interaction assays*

- *New accurate way of determining Mark-Houwink constants for the viscometric determination of molecular weight of chitosan over a wide range of molecular weights and degrees of acetylation*

- *Dilute solution properties of chitosan and its conformational changes across a wide range of copolymer compositions*

- *Dilute solutions properties and its implications on molecular conformation in solution for a recently patented anionic chitosan polymer soluble at neutral pH*

Previous dissertations

- *Structure-property relationships in chitin-based reverse osmosis membranes,* The Nottingham Trent University/Georg-Simon-Ohm-Fachhochschule Nürnberg, 1996

- An investigation of flocculation of reactive and acid anionic dyes with chitosan and metachromatic properties of several direct dyes, The Nottingham Trent University/Georg-Simon-Ohm-Fachhochschule Nürnberg, 1994

Advan. Chitin Sci., Vol.4

M. G. Peter, A. Domard and R. A. A. Muzzarelli, eds.
University of Potsdam, 2000. ISBN 3-9806494-5-8

Syneresis aspects of chitosan based gel systems

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Summary
Chitosan (Hirano) gels were produced by homogenous *N*-acylation of chitosan in acyl-anhydride/acetic acid/co-solvents systems. The syneresis behaviour was investigated and monitored for up to 300 days. The effects of acyl anhydride concentration and co-solvent (diethyl ether):methanol ratio were studied. It was found that the addition of ether as a second co-solvent in the acetic acid/methanol system increased the reaction efficiency so decreasing the amount of acyl-anhydride necessary for gelation. The influence of the head-space volume on the rate of syneresis was also investigated.

Introduction
Chitosan can be homogeneously acylated to, for example, chitin in an acetic acid/methanol (MeOH) system with the respective anhydride as the reagent and the reaction system undergoing gel formation [1][2]. During the homogenous *N*-acylation of chitosan it was observed that gels prepared with a second co-solvent, ether, were much clearer than equivalent Hirano gels. The gels also exhibited much less syneresis than expected or has been reported in the literature [3]. One sample remained as a clear gel over a period of 18 months.

Materials and Methods
The chitosans employed were
a) Aber Technologies CHITOSAN MV – (Chit 98) Ref: A32E03 – (medium viscosity) le 06/07/95 F_A=[0.01], 65 cps (1% chitosan in 1% acetic acid) and
b) A sample of chitosan (low viscosity) prepared in the laboratory from Indian crab, F_A=[0.28], 16 cps (1% solution in 1% acetic acid).

Chitosan a) was used unless otherwise stated.
They were purified by filtration and reprecipitation.
General grade reagents and solvents were used as supplied.

Preparation of *N*-acetyl chitosan
For the *N*-acetylation of chitosan a 1 % polymer solution in 0.1M acetic acid was diluted 1:1 with MeOH and acetic anhydride was added in molar proportions ranging from 0.8 to 6.0 based on free amine groups [1][2]. The mixtures were prepared on stirring readily in appropriate moulds and covered with laboratory film unless otherwise stated.

Monitoring of syneresis

The weight of the initial system was determined after mixing. After a period of time the exuded solvent was discarded and the gels were carefully padded dry with laboratory tissue and weighed. The gels were not cut loose from the moulds as it has been shown [3] that the difference in syneresis of a loose gel compared to an attached gel levels off in less than 24h.

Determination of Degree of acetylation

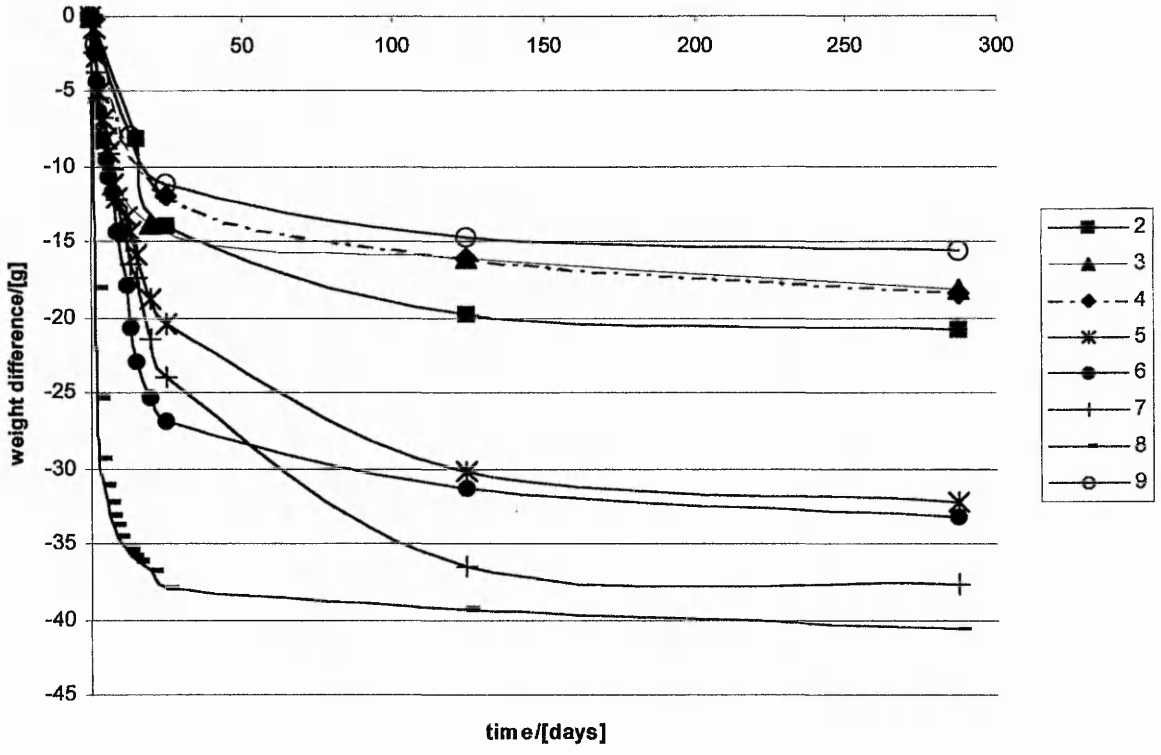
The degree of acetylation was determined via dye adsorption [4].

Results and discussion

Gels series 0: Anhydride/amine proportion varying from 0.85 to 6.00

A series of 8 *N*-acetylation systems plus one reference system without reagent (acetic anhydride) was prepared using ether and the same solvent proportions as in the initial quantitative observation. In this series the effect of varying the proportions of anhydride to amine groups in a range of 0.85 to 6.00 (see table 1) on the syneresis behaviour of the gels was looked at. As to be expected reference sample 9 without reagent showed the least weight loss, since there are only evaporation but no syneresis effects. Sample 1 did not gel and was thus discarded. While samples 2-4 show rather inconclusive behaviour* and no clear trend can be made out, there is a distinct increase in weight loss between the former group of samples and group 5 – 8. The latter group also shows a clear trend of increasing syneresis with increasing anhydride proportions.

Figure 1: range 0 - varying anhydride concentration



* This group synerised in the region of lower weight losses, which might have compromised the accuracy of the determination of differences in weight.

Table 1: composition of series 0 and C

series 0 and C	1	2	3	4	5	6	7	8	9
chitosan solution/[g]*	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
MeOH/[ml]	18.4	17.6	16.7	16.1	15.5	14.9	14.3	8.6	20
ether/ml	10	10	10	10	10	10	10	10	10
Ac_2O/MeOH 1:10 / [ml]	1.6	2.4	3.3	3.9	4.5	5.1	5.7	11.4	-
molar proportion Ac_20/-NH_2	0.84	1.26	1.74	2.05	2.37	2.68	3.00	6.00	0.00
approx. (intended) proportion**	0.85	1.25	1.75	2.00	2.25	2.75	3.00	6.00	0.00
F_A for series 0		0.87	0.94	0.95	0.96	0.97	0.97	0.99	
F_A for series C			0.88	0.89	0.92	0.94	0.94	0.96	

*[medium viscosity (series 0)/low viscosity (series C)] chitosan
** 0.85 was chosen, since formation of a firm gel starts at approx. 80% acylation [5]
** 6.00 was chosen, since it has been reported [6] that syneresis in chitin gels reaches an equilibrium at above ratio

Gels series A: ether/MeOH proportion varying from 0.2 to 2

The influence of the ether concentration of the gelation systems was investigated by varying the ether/MeOH ratio from 1:5 to 2:1 (see table 2). No gelation occurred for a ratio >1:1 (gel 7) and gelation occurred readily at 1:5 but not at 1:3. Gels 3 to 5 show an increase in weight loss over time with increase in ether. Gel 1's weight decrease however is situated between gel 3 and 4. Gel 6 only formed a very slight gel and showed comparatively little syneresis, both indicating low cohesion forces within the gel and thus low ridgidity and contraction.

Figure 2: range A – varying co-solvent ether concentration

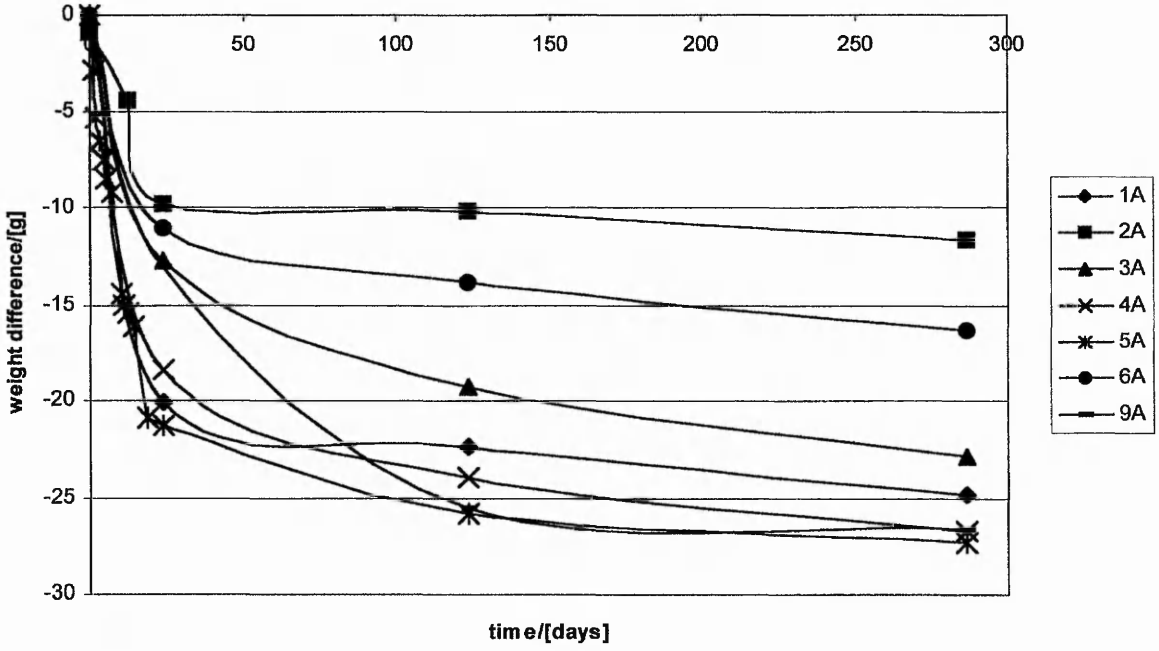


Table 2: composition of series A

series A	1	2	3	4	5	6	7	8
chitosan solution/[ml]	30	30	30	30	30	30	30	30
MeOH/[ml]	20.5	18.5	16.5	14.5	12.5	10.5	8.5	5.5
ether/ml	5	7	9	11	13	15	17	20
Ratio ether/MeOH	1:5	1:3	1:2.3	1:1.7	1:1.3	1:1	1:0.8	1:0.5
Ac_2O/MeOH 1:10 / [ml]	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
F_A	0.94		0.95	0.96	0.92	0.88		
gelation	+	-	+	+	+	(+)	-	-

Gels B: varying head-space volume

The effect of head-space volume was looked at and the extend of syneresis of chitin gels with co-solvent ether was compared to Hirano gels. A matrix of gels was prepared of which groups of 3 each were prepared with ether/MeOH proportions 0:1 (=[*]), 0.4:1 (=[']) and 0.8:1 (=[~]). One of each group was left uncovered (nc), covered with cling film (cf) and covered with laboratory film (pf) (see table 3).

The weight loss of the gels is highest when uncovered and lowest when covered with laboratory film. When comparing the weight loss differences between the groups of different ether content it shows that the differences are narrowed down with increasing ether content. While the syneresis of the uncovered gels is fairly uniform across the range, the weight loss of the covered gels increases considerably with increase in ether concentration. When comparing the groups of gels with equivalent covering there is a surprising trend in the group of uncovered gels. Unlike the covered gels, which lose more solvent with higher ether content, the uncovered gels lose more solvent with decreasing ether content (see figure 3 (a, b, c)). An explanation for this observation would be, that contraction due to fast evaporation of ether-enriched solvent seals off the pores at the gel-gas phase boundary. This is not the case, when the gel is covered with film since the head-space volume is limited. An evaporation equilibrium is therefore reached and the gel surface stays in contact with solvent vapour phase.

Figure 3a: uncovered gels

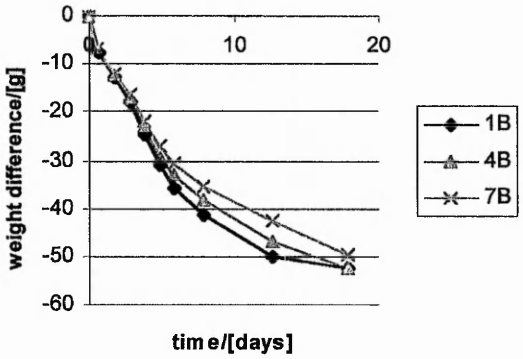


Figure 3b: gels covered with cling film

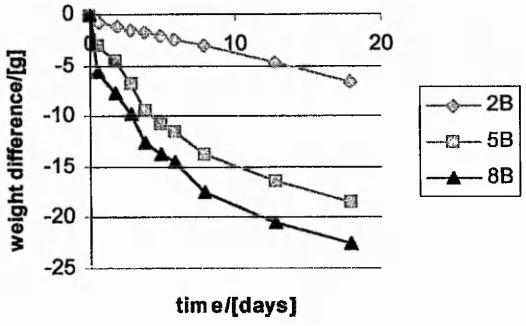


Figure 3c: gels covered with laboratory film

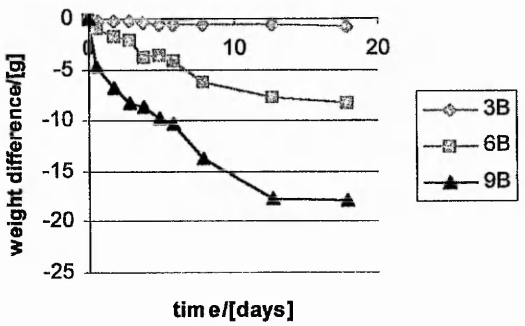


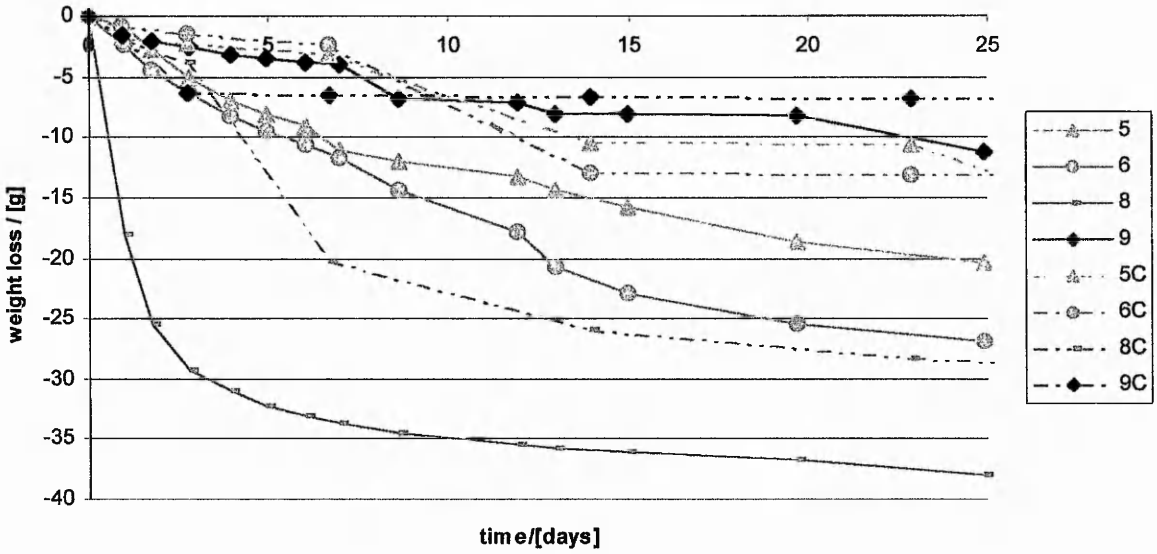
Table 3: composition of series B

series B	1	2	3	4	5	6	7	8	9
chitosan solution/[ml]	30	30	30	30	30	30	30	30	30
MeOH/[ml]	26.7	26.7	26.7	17.7	17.7	17.7	13.7	13.7	13.7
ether/ml	0	0	0	9	9	9	13	13	13
Ac_2O/MeOH 1:10 / [ml]	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
covering	nc	cf	pf	nc	cf	pf	nc	cf	pf
F_A	0.87	0.87	0.88	0.91		0.92	0.91	0.92	0.92

Gels C: effect of molecular weight

A series of gels equivalent to series 0 was prepared using lower molecular weight chitosan in order to see how that would affect syneresis. System 1C and 2C did not show gelation, although the starting F_A was higher than in series 0.

Figure 4: comparison of gels from two different MW chitosans



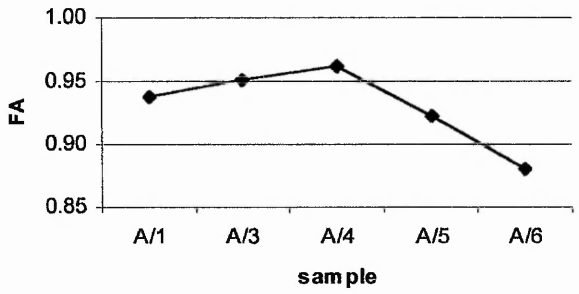
The gels, with an exception of 4C, showed less syneresis than the analogous ones in series 0. This can be seen in Figure 4. Looking at the data for 9/9C the latter shows less solvent

loss. This indicates a decrease of boiling point of the solvent system due to the increase in particles in the systems with lower MW chitosan. However the difference in weight loss between gels */*C is considerably greater and this is due to syneresis. The longer chain chitosan molecules possess higher intermolecular forces and thus the contraction of the gels with higher MW chitosan is greater.

Effect of co-solvent ether on reaction and gelation of Hirano gels

It was observed, that gelation occurred at lower acetic anhydride concentrations in the presence of ether. While system 3 formed a rigid gel, an equivalent gel prepared without ether only formed a very light gel indicating either higher acetylation or an influence of ether on the gel formation. Looking at the F_A values of series B (table 3) one can see that gels 1B-3B are lower than those of 4B-9B, which were prepared with ether. The F_A values of series A show maximum for ether/MeOH ratio of 1:1.17. This again suggests that the presence of ether makes the reaction more efficient and also, that there is an optimum ratio. This is a very promising result and is intended to investigate this further in the future.

Figure 5: degree of acetylation of series A



Acknowledgements

One of the authors (RD) wishes to thank TNTU for the award of a research bursary for the period during which the above work was carried out.

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Presented at: *6th European Training Course on Carbohydrates*
Carbohydrate Research Foundation, The Hague, The Netherlands
Lajos Kossuth University, Debrecen, Hungary, 2000

Aspects of chitosan-dye interactions: flocculation and metachromasy

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Summary

- Chitosan and some derivatives as a flocculant for dyes
- Flocculation of 3 reactive dyes at different dye and NaCl concentrations
- Flocculation of 3 acid dyes with up to 3: influence of number of sulfonic acid groups
- Results by
  - Beer-Lambert (absorbance)
  - Kubelka-Munk (colour strength - CS)
- Metachromasy of structurally related compounds

Introduction

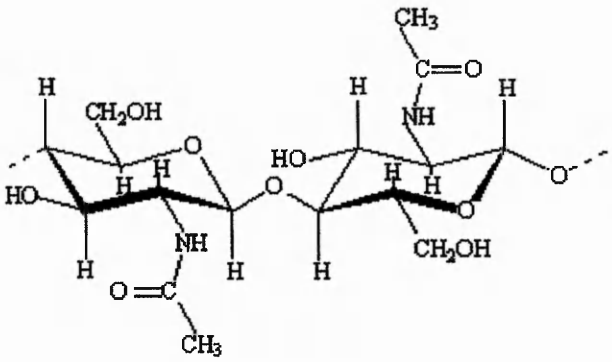


Figure 1a: Idealised structure of chitin

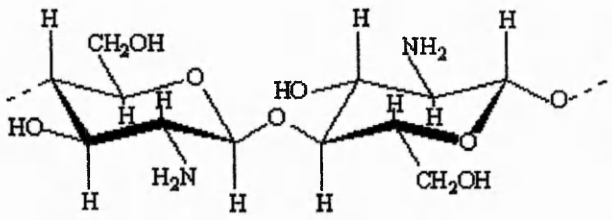


Figure 1b: Idealised structure of chitosan

Physical chemistry of flocculation

Dissolved species: stabilised in solution by double layer (Stern Layer and diffuse layer) of solvent dipoles and counterions; needs to be overcome for aggregation

Coagulation: Reduction of net electrical repulsive forces at particle surfaces allows them to aggregate

Flocculation: Involves chemical bridging between dissolved species

Restabilisation: hypercompensation of solute charge by excess of flocculant

What makes chitosan an interesting flocculating agent?

- renewable resource
- polyelectrolyte
- high charge density
- cationic
- produced from the second most abundant organic compound on this planet
- soluble in dilute acids
- lower toxicity than sugar or salt[1]

Monitoring of Flocculation

The residual dye concentration was determined by absorbance and colour strength (CS). Colour strength was employed, in addition to measurement at single wavelength since some dyes exhibited metachromasy (spectral shift of λ_{max}) in the flocculation medium and CS can be used directly at higher dye concentrations.

Absorbance

Beer-Lambert-Law

$A = \varepsilon * c * l$

A=Absorbance
ε=Absorption (Extinction) Coefficient/[$dm^3 mol^{-1} cm^{-1}$]
c=Concentration/[mol dm^{-3}]
l=path length of cell/[cm]

Colour strength (CS)

Kubelka-Munk equation

$$\frac{K}{S} = \frac{(1-r)^2}{2*r}$$

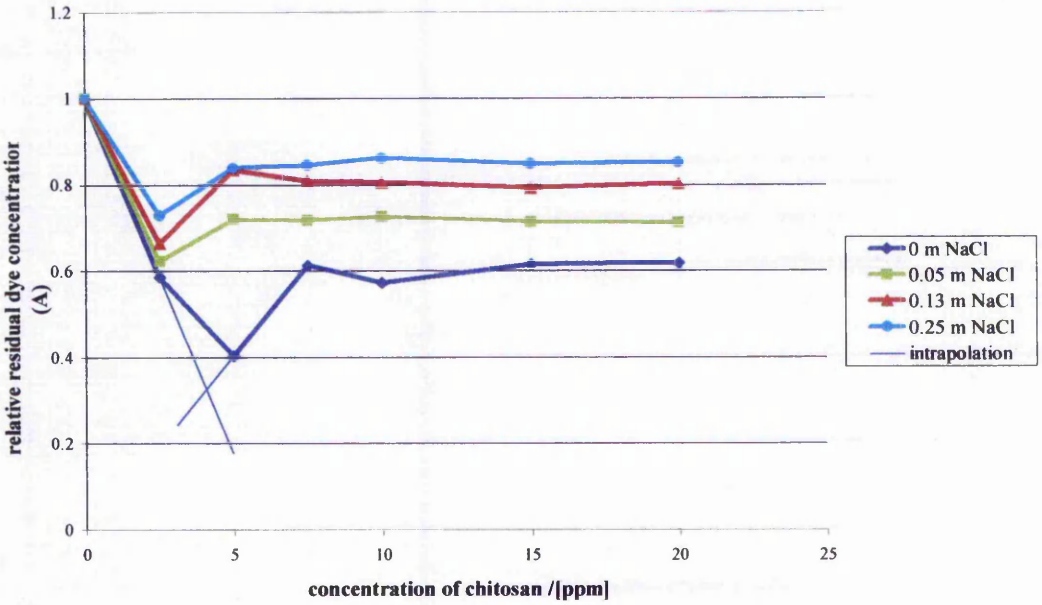
r=transmittance (or reflectance)

$$CS = \frac{\Sigma \frac{K}{S}(Batch)}{\Sigma \frac{K}{S}(Standard)}$$

Reflectance (or transmittance) determined at 16 wavelengths, normally over the range 400 and 700nm

Results and discussion

Unless otherwise stated the relative dye concentration is derived from absorbance.



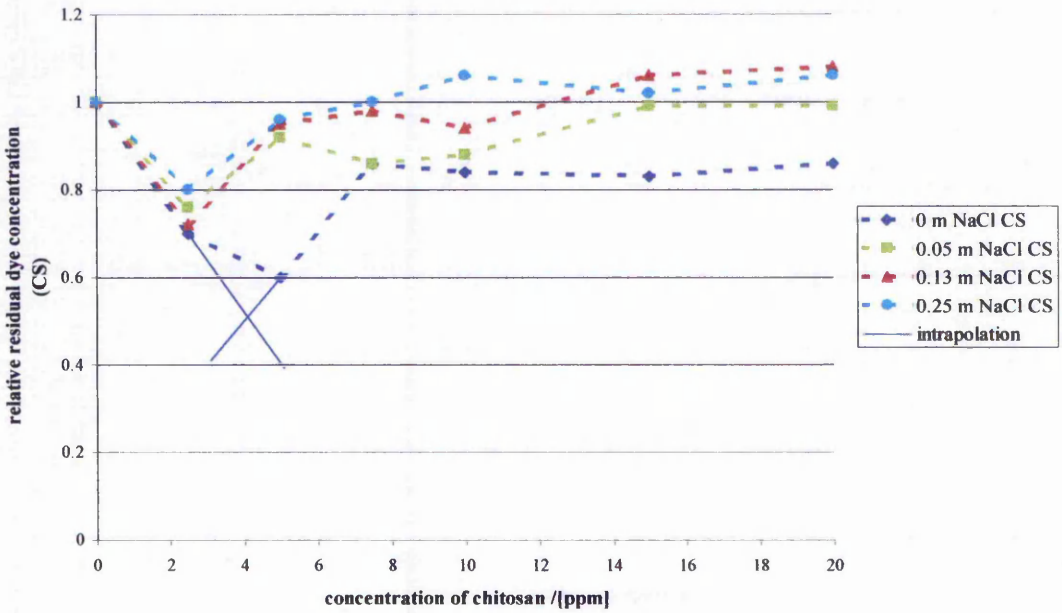


Figure 2a and 2b: Typical examples of flocculation curves for reactive dyes at low dye concentrations derived from absorbance and CS respectively

The dye concentration decreases with increase in chitosan present down to the minimum, after which restabilisation due to overdoses of flocculant occurs very quickly. In the presence of increasing NaCl concentrations the minimum shifts to higher values of residual dye.

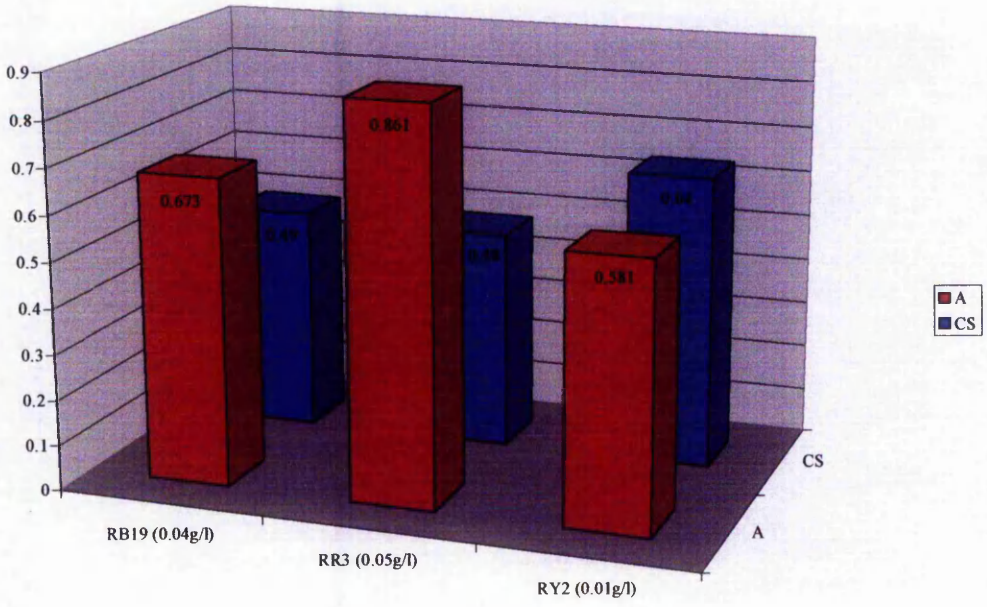


Figure 3: Comparison of the fraction of dye removed for 3 different dyes
The dye removed ranges between 58% and 86% from absorbance determination and 48% and 64% from CS determination. The former are bound to be seemingly higher due to metachromasy. A trend to more efficient removal at higher concentrations is visible.

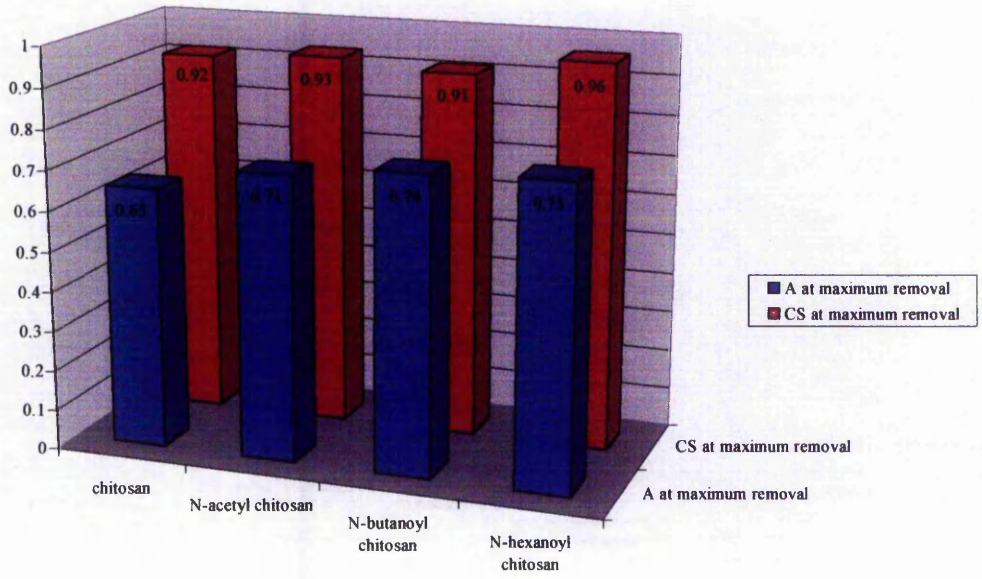


Figure 4: Influence of substituent size at a fraction of substitution of approx. [0.2] on the flocculation behaviour of AR88 (0.07g/l)
The influence of substitution is only marginally in favour of larger substituents. The restabilisation behaviour could not be improved.

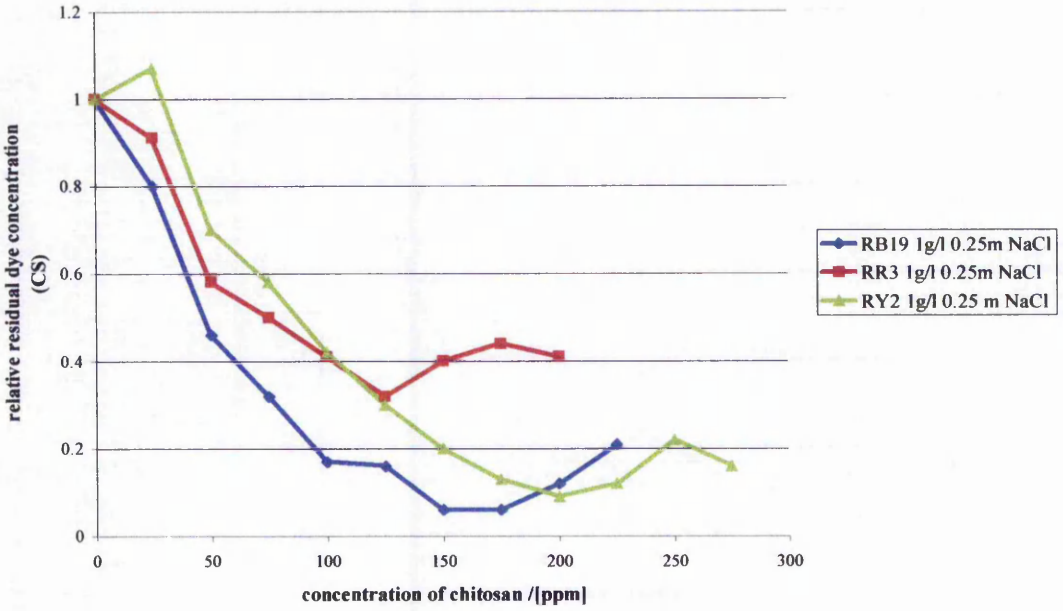


Figure 5: Flocculation of 3 reactive dyes at dye concentration simulating residual dye bath concentration in the presence of 0.25m NaCl

At these higher concentrations the dye removal by flocculation with chitosan is very efficient with the percentage of removed dye ranging between 68% and 94%. Restabilisation occurs much less rapidly after maximum dye takeout.

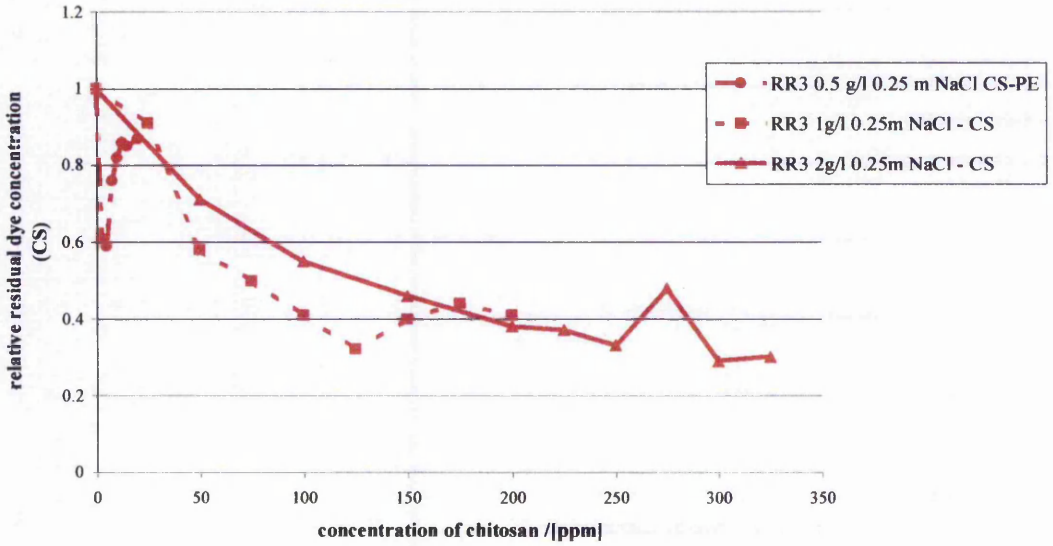


Figure 6: Flocculation of RR3 at different dye concentrations in the presence of 0.25m NaCl

The dye removal is more efficient at higher dye/flocculant concentrations. The dye take out increases from 41% to 68 (71)% when the dye concentration increases from 0.5g/l to 1 (2)g/l. The

increase of dye concentration from 1g/l to 2g/l does not have an effect on the dye removal, however overdosing becomes less of a problem the higher the dye concentration is, as the restabilisation slope becomes significantly lower. While overdosing the flocculant by <10ppm for the dye at 0.5g/l leads to a restabilisation of 50%, the same change only leads to restabilisation of <1% for the dyes at >1g/l.

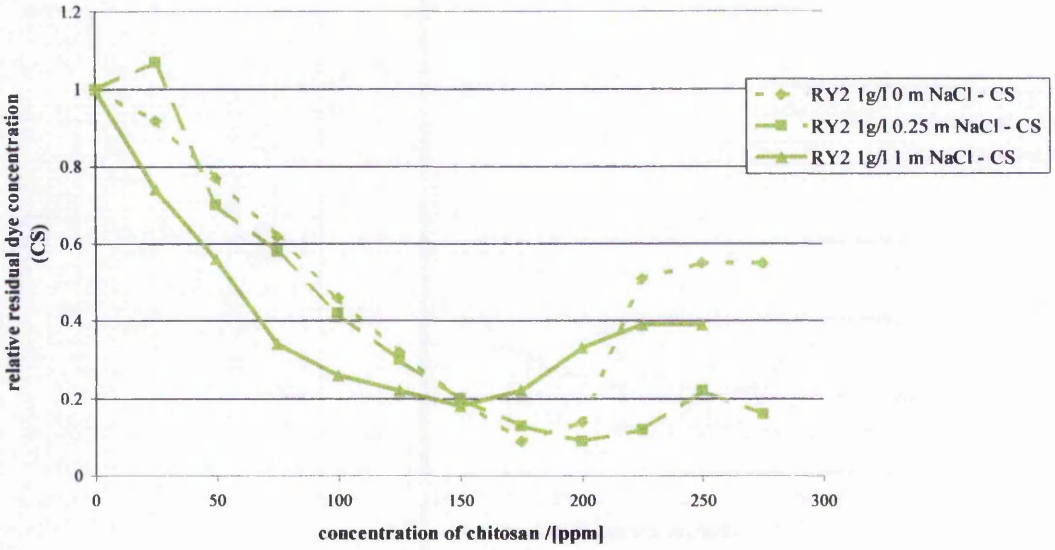


Figure 7: Influence of NaCl concentration similar to dye baths on the flocculation behaviour

At a dye concentration of 1g/l the influence of NaCl in the medium is much less pronounced as at lower concentrations (see figure 2a and 2b). A concentration of 1m NaCl only leads to a decrease in removal of <10% compared to 0m and 0.25m NaCl.

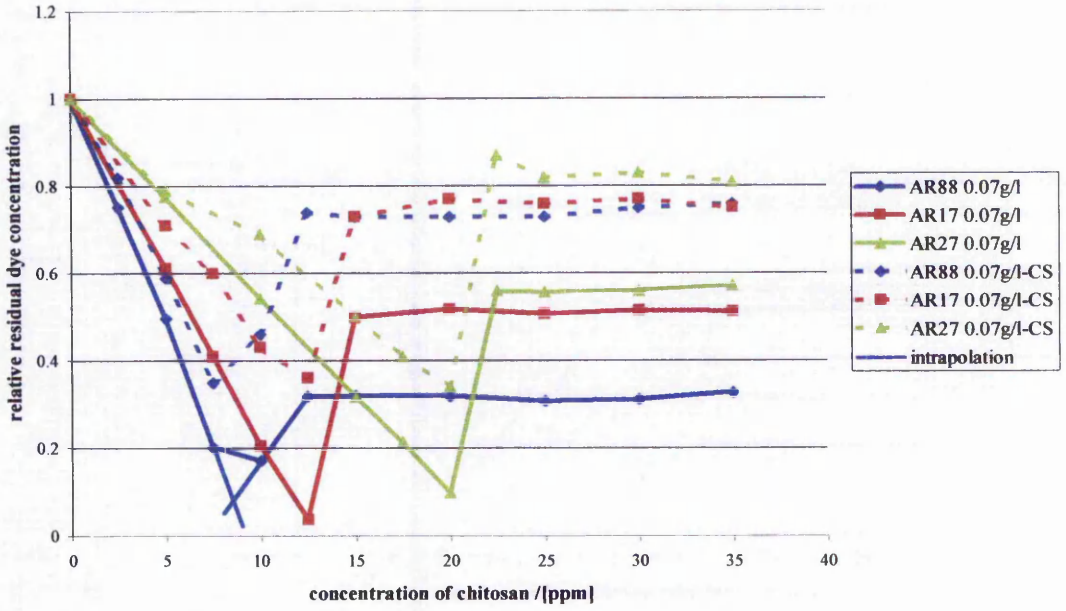


Figure 8: Flocculation of 3 acid dyes with 1 to 3 sulfonic acid groups
The relationship between the number of sulfonic acid groups and the amount of chitosan required was approximately linear.

Dye	AR88	AR17	AR27
Number of sulfonic acid groups	1	2	3
Ratio of chitosan required (absorbance)	1	2.19	3.00
Ratio of chitosan required (CS)	1	2.14	3.00
Reported[2]/approx.	1	2	3

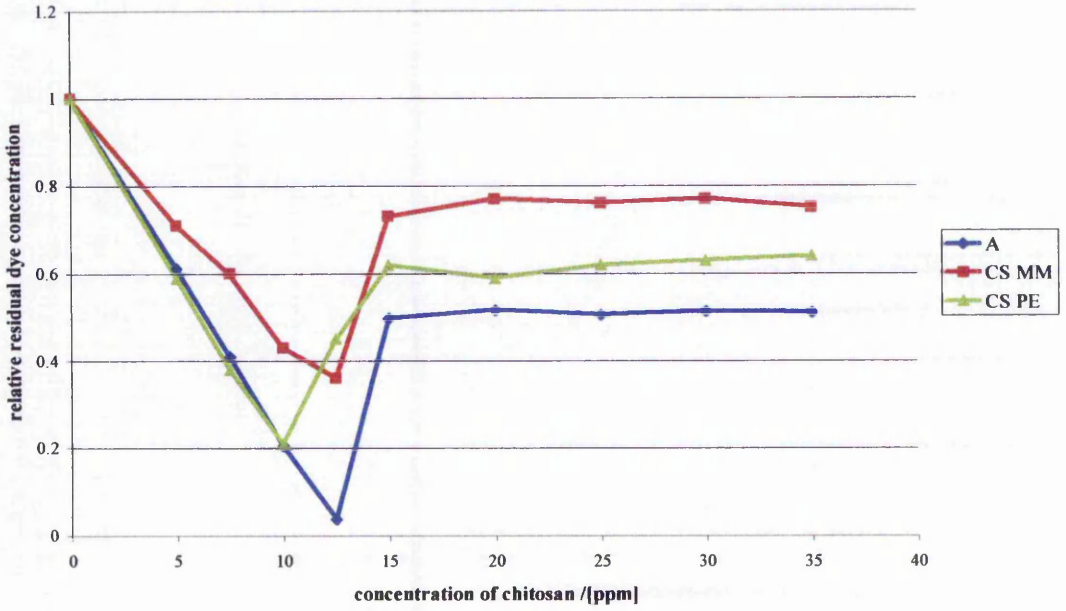


Figure 9: Comparison of three different methods of measuring residual dye for AR17 (0.07g/l)

The three different sets of values were obtained by absorbance and transmittance measurements on a *Perkin Elmer Spectrophotometer* and reflectance measurement on an *ICS Micro Match*. Absorbance values can be deceptively low due to metachromatic shifts of λ_{max}, while the accuracy of the reflectance values at low dye concentrations may be compromised. The true value, as the values for CS obtained from transmittance measurements suggest to be in between the two.

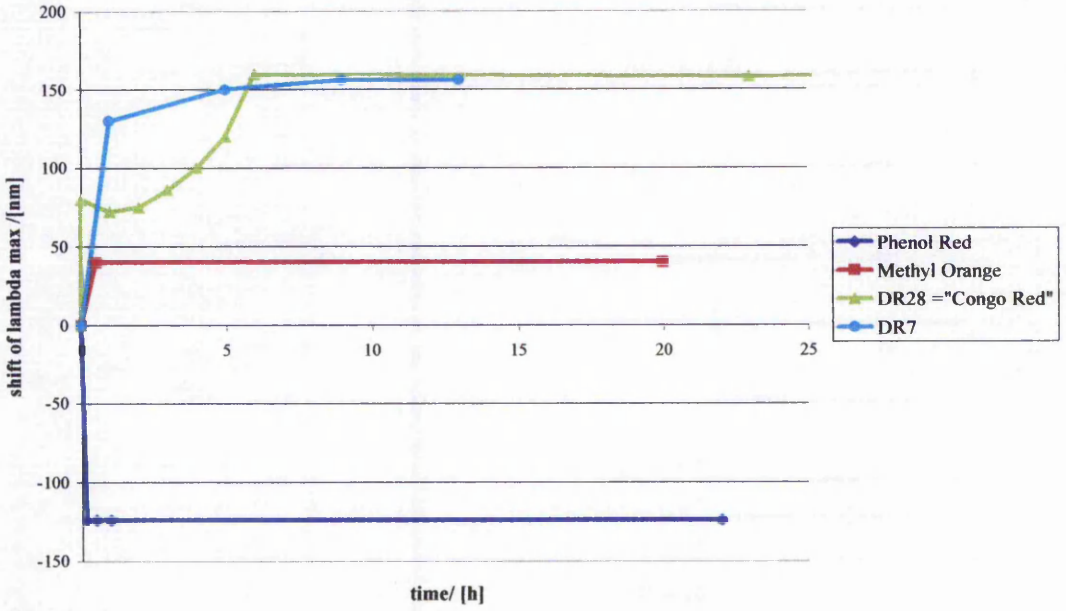


Figure 10: Metachromatic behaviour of 2 direct dyes compared to 2 indicators in 0.04m acetic acid

In the presence of acetic acid several dyes showed metachromatic shifts of λ_{max}, which leads to a decrease of absorbance at the initial wavelength of λ_{max} and seemingly lower concentration values.

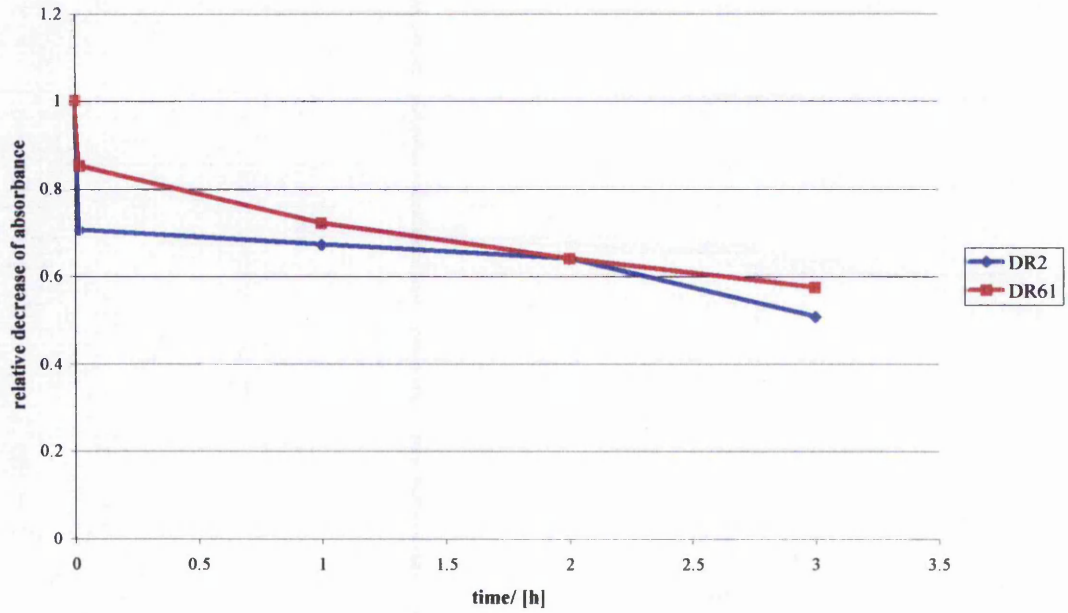


Figure 11: Hypochromic behaviour of 2 direct dyes in 0.04m acetic acid

In the presence of acetic acid the above dyes exhibited hypochromy, which means, that despite the fact that λ_{max} is constant the absorbance measured is time dependent.

Conclusions

- Flocculation by chitosan is very effective at higher dye concentrations as would be found after pre-concentration by other methods or in some exhausted dyebaths
- NaCl content effects flocculation unfavourably at lower dye concentrations but only negligibly at higher (dye bath) dye concentrations
- The relationship between anionic groups on the dye molecule and the amount of chitosan required for maximum removal is linear
- Monitoring by absorbance is suitable as a facile method for the determination of the end value of the chitosan-dye titration, but gives deceptively low values for the amount of residual dye due to metachromatic behaviour of a number of dyes in the medium (chitosan, acetic acid)
- For determination of the absolute values of residual dye other methods such as CS determinations need to be employed

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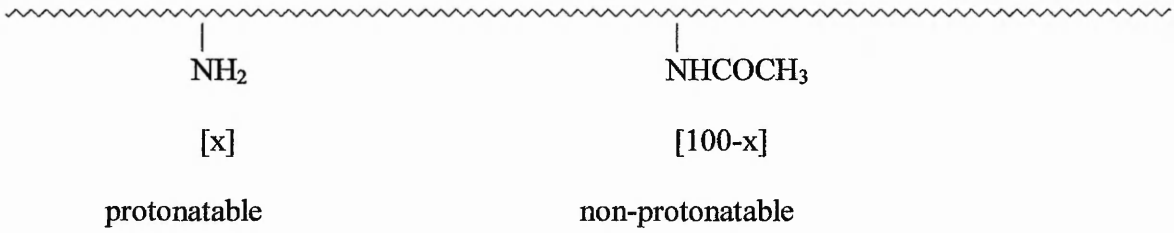
DETAILED DERIVATIONS AND EXAMPLES FOR DERIVED AND EMPLOYED EQUATIONS

Determination of the degree or fraction of substitution for secondary substitution from experimental EWs

EW and degree of acetylation for chitin and chitosan



$$EW = \frac{x*161+(100-x)*203}{x}$$

$$EW*x = 161*x + 20300 - 203*x$$

$$20300 = (EW+42)*x$$

$$\Rightarrow x = \frac{20300}{EW+42}$$

$$D_A = 100 - x$$

$$D_A = 100 - \frac{20300}{EW+42}$$

$$D_A = \frac{100*(EW+42)-20300}{EW+42}$$

$$D_A = \frac{EW+42-203}{EW+42}*100$$

$$D_A = \frac{EW-161}{EW+42}*100$$

$$\Rightarrow F_A = \frac{EW - 161}{EW + 42}$$

EW = equivalent weight of amine group

x = percentage of glucose amine units

D_A = degree of acetylation

F_A = fraction of acetylated sugar units

EW and substitution for non-protonatably substituted chitin and chitosan with known Da=a

Deriving from the principle applied for chitin and chitosan the degree of secondary substitution for a non-protonatable secondary substituent can be determined. The varying contribution which substituents with a size different from that of the acetyl group would make to the weight of the material and the EW respectively have to be considered. The formula is not specific to acyl derivatives, but can be employed for any other non-protonatable* group with S = formula weight of the respective substituted sugar unit.

NH_2	$NHCOCH_3$	NHCOR
[x]	[a]	[100-x-a]
protonatable	non-protonatable	non-protonatable

$$EW = \frac{x*161 + a*203 + (100 - a - x)*S}{x}$$

$$EW*x = 161*x + 203*a + 100*S - S*a - S*x$$

$$EW*x = 161*x - S*x + 203*a + 100*S - S*a$$

$$EW*x - 161*x + S*x = 203*a + 100*S - S*a$$

* Obviously the protonatability of the substitutent in question is referring to the conditions under which the EW is experimentally determined. A substituent may well show differing behaviour under various conditions.

$$(EW-161+S)*x=(203-S)*a+100*S$$

$$\Rightarrow x=\frac{(203-S)*a+100*S}{EW-161+S}$$

for S=259 (*N*-hexanoyl chitosan)

$$\Rightarrow x=\frac{(203-259)*a+25900}{EW-161+259}=$$

$$=\frac{-56*a+25900}{EW+98}$$

for x = 100

a = 0

EW = 161

(not defined for x=0)

$$\Rightarrow x=\frac{25900}{161+98}=\frac{25900}{259}=100$$

$$D_S=100-x$$

$$F_S=\frac{Ds}{100}=1-\frac{x}{100}$$

$$F_N=F_S-F_A$$

EW = equivalent weight of amine group

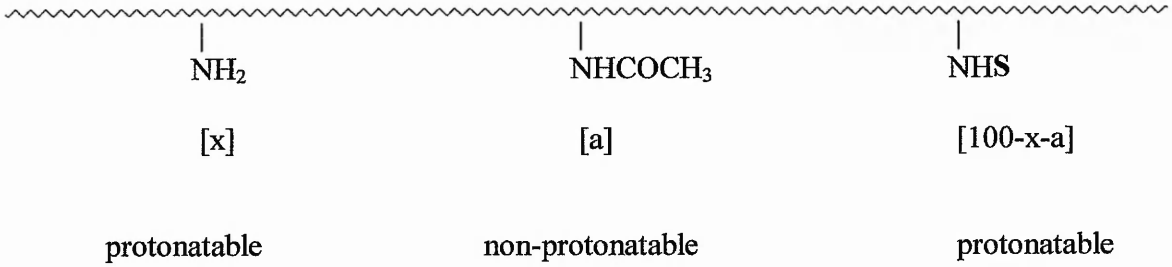
x = percentage of glucose amine units

D_S = overall degree of substituted sugar units

F_S = overall fraction of substituted sugar units

D_A = percentage of acetylated glucose amine units (determined before)

F_N = fraction of secondary substituted sugar units



Comment:

When the EW is determined experimentally for a specific sample, rather than calculated for theoretic curves, then the fraction of all protonatable amine groups will be determined, as these will be the interacting species in metachromatic and dye adsorption processes. This explains, why in the following formula the denominator consists of the percentage of all protonatable groups and the numerator is the sum of the products of the molecular weight of each occurring sugar unit and the respective percentage in which they occur along the polymer chain. [x] occurs in the numerator, as this determines the contribution of glucoseamine groups to the MW of the polymer, while it does not appear in the denominator, as the latter consists of all protonatable groups, which also includes the [100-a-x] portion being the percentage of secondary substituents. In the final result, when an experimentally determined EW is employed to solve the equation and determine the degree of substitution, [x] is the percentage of all protonatable amine groups, in this case the sum of $-NH_2$ and -NHS.

$$EW = \frac{x*161 + a*203 + (100-a-x)*S}{100-a}$$

$$EW*(100-a) = x*161 + 203*a + 100*S - a*S - x*S$$

$$EW*(100-a) = (161-S)*x + (203-S)*a + 100*S$$

$$EW*(100-a) - (203-S)*a - 100*S = (161-S)*x$$

$$x = \frac{EW*(100-a) - (203-S)*a - 100*S}{(161-S)}$$

for x = 100
a = 0
EW = 161

(not defined for x=0)

$$x = \frac{161*100 - 100*277}{161 - 277} = \frac{100*(161 - 277)}{(161 - 277)} =$$

$$= 100$$

Different formulas for the experimental and calculative determination of EW

Metachromatic titration

$$EW = \frac{w*V}{10*c}$$

EW = equivalent weight of amine group
w = oven dried weight of polymer in grams
V = volume of chitosan solution at the equivalence point /[mL]
c = concentration of the original stock dye solution
(determined accurately by absorbance, ε_{max}=22500 cm^2 mol^{-1} (211))

Dye adsorption

$$EW = \frac{w*\varepsilon\max}{\Delta A*f*V}$$

EW = equivalent weight of amine group
w = oven dried weight of polymer in grams
ε_{max} = extinction coefficient
ΔA = difference in absorbance values of blank dye solution and test dye solution
f = dilution factor (typically 100)
V = volume of dye aliquot /[L]

For known degrees of substitution:

$$EW = \frac{(F_A * mw) + ((100 - F_A) * ch)}{100 - F_A}$$

EW = equivalent weight of amine group

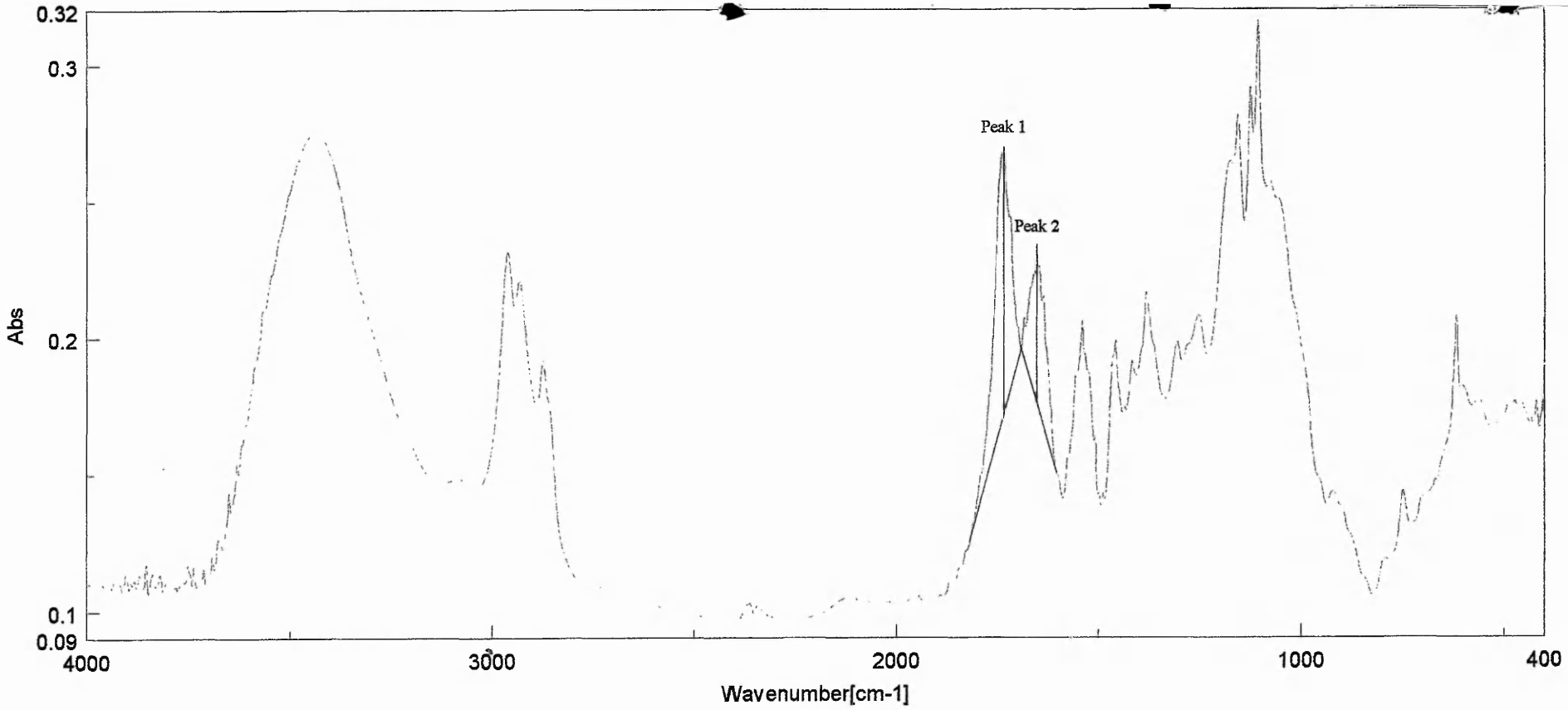
FA = fraction of acetylated sugar units

mw = molecular weight of substituted repeat unit (203g/mol for chitin)

ch = molecular weight of the monomeric unit of polyglucosamine ($161 gmol^{-1}$)

Example:chitosan [0.20]

$$EW = \frac{(15 * 203) + (15 * 161)}{85} = 196.82 g / mol$$



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Sample Name	B2H1
Comment	13-11-01

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Apodization	Cosine
Scanning Speed	2 mm/sec

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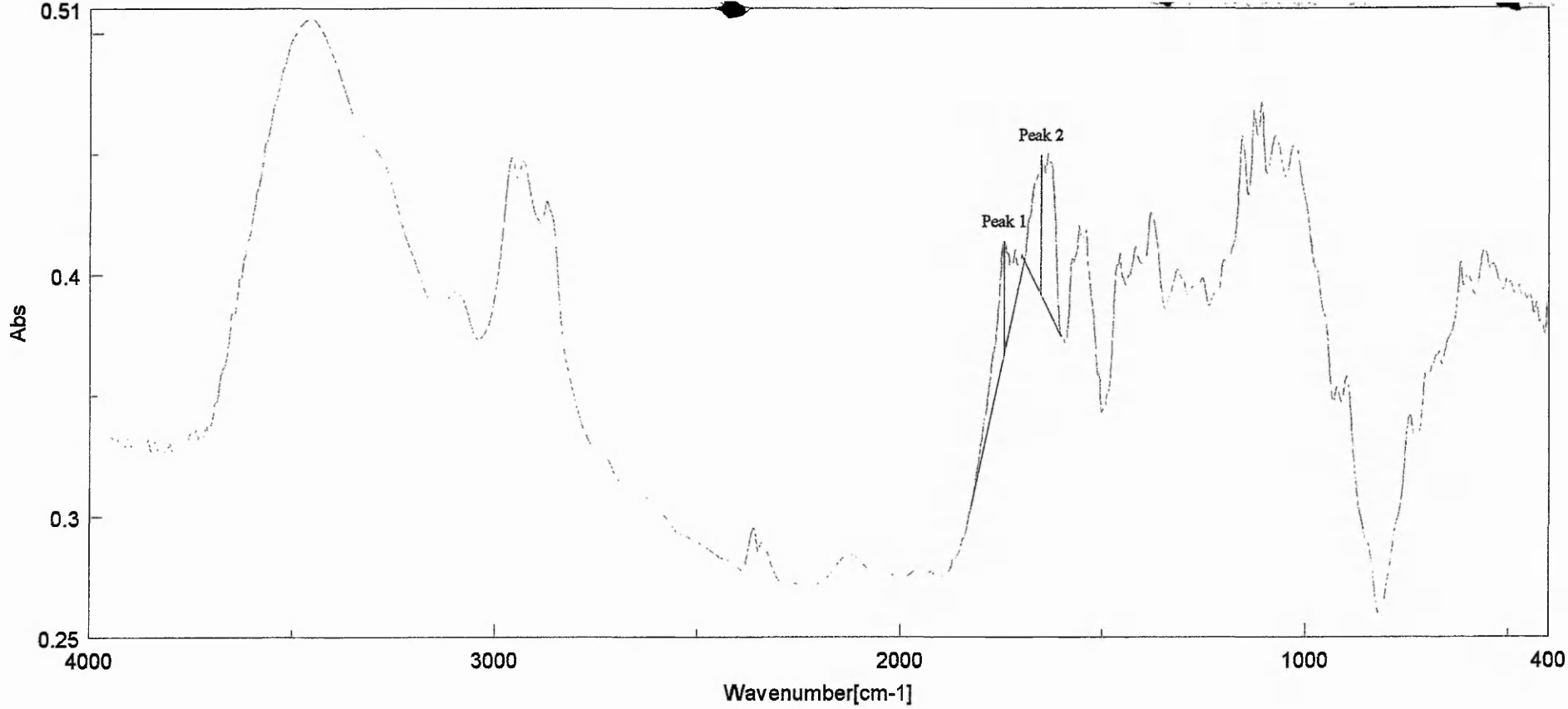
Peak 2	1653.66	1605.45	1696.09	0.0579	0.5882



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