

İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**INFLUENCE OF CONFORMATION OF HYALURONIC ACID IN SOLUTION
ON PROTON MOBILITY**



M.Sc. THESIS

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Department of Chemistry

Science of Chemistry Programme

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**HYALÜRONİK ASİT'İN ÇÖZELTİDEKİ KONFORMASYONUNUN
PROTON HAREKETLİLİĞİNE ETKİSİ**

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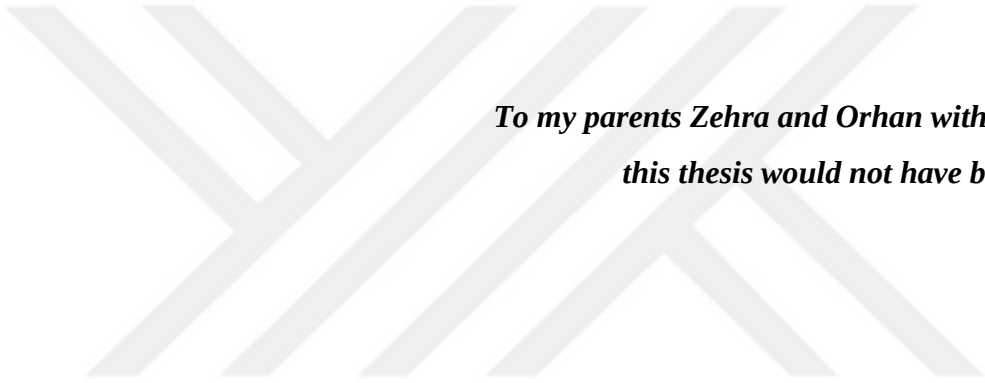
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*To my parents Zehra and Orhan without whom,
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ABBREVIATIONS

¹H-NMR	: Proton Nuclear Magnetic Resonance
DLS	: Dynamic light scattering
DMTMM	: 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium
DS	: Degree of substitution
DSC	: Differential Scanning Calorimetry
HA	: Hyaluronic acid
HM_w	: High molecular weight
HPC	: Hydroxypropyl cellulose
LM_w	: Low molecular weight
M_w	: Molecular weight
NMMNO	: N-methyl morpholine N-oxide monohydrate
PBS	: Phosphate buffer solution
SLS	: Static Light Scattering



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INFLUENCE OF CONFORMATION OF HYALURONIC ACID IN SOLUTION ON PROTON MOBILITY

SUMMARY

Hyaluronic acid (HA) is polysaccharide made of D-glucuronic acid and N-acetyl glucosamine repeating disaccharide units. The molecular weights of HA from different sources are highly variable, ranging from 10^4 to 10^7 Da. HA is in the form of sodium salt in the physiological conditions, hence negatively charged and shows polyelectrolyte properties. It is essential component of tissues in the vertebrate organism and found in the rooster comb, human umbilical cord, synovial fluid and eye bovine. Furthermore, almost half of the human body's HA occurs intracellularly in skin. Traditionally HA extracted from rooster comb, however, due to existence of the protein impurities this processes is facing growing concern. Hence, bacterial fermentation is emerged as an alternative method for HA production and now mainly bacterial production is used.

The high molecular HA has unique properties like moisturising retention ability and viscoelasticity, coupled with its lack of immunogenicity and toxicity. These properties provides a wide range of application areas such as aesthetic medicine, arthrology, ophthalmology, oncology and wound healing. For increase the residence time of HA in vivo is necessarily modified HA can be used. It is really significant to determine exact structural information about the HA. Infrared spectroscopy, ninhydrin assay and nuclear magnetic resonance spectroscopy are the methods used for structural characterization. Among these methods hydrogen nuclear magnetic resonance (^1H -NMR) spectroscopy is the most common method due to easy sample preparation and short analysis time. However, it is not easy to perform solution ^1H -NMR analysis of HA because as all electrolytes HA chains interact with each other even at dilute solution. This behaviour produces highly viscous solutions at very low concentrations, also affects the proton mobility and cause broad signals in spectra which is not easy to assign.

Highly viscous HA aqueous solutions show gel like behaviour. Gels consist of mobile and immobile regions. With solution ^1H -NMR only protons of mobile region are visible, for characterize immobile region solid state ^1H -NMR must be used. Immobile region of HA is proved by solid state ^1H -NMR; acetyl, sugar backbone, anomeric protons are determined. It is possible to turn the immobile region to mobile one by changing ionic strength and pH. By changing these two parameters the proton mobility is investigated and a correlation between proton mobility and the conformation of HA in solution is found.

In this study, high molecular weight microbial HA ($1,5 \times 10^6$ g/mol) is investigated. Solid HA is dissolved in the reagent solutions as 0,075 M, 0,125 M, 0,5 M, 1 M, 2 M sodium chloride (NaCl), 0,071 M, 0,125 M, 0,25 M, 0,5 M, 1 M sodium hydroxyde (NaOH), 0,25 M, 1 M, 4 M urea, 0,5 M sulfuric acid (H_2SO_4) and 0,5 M hydrochloric acid (HCl). For proton mobility determination, 4 mg/mL HA in reagents solutions with deuterium oxide are prepared, measured by ^1H -NMR and

from the obtained spectra proton mobility is calculated. Radius of gyration (R_g) and hydrodynamic radius (R_h) of HA in solutions are obtained by light scattering technique, intrinsic viscosity ($[\eta]$) is by viscosimetric measurements.

Determined R_g , R_h , $[\eta]$ values were plotted against proton mobility. It has been shown, that there is inverse correlation, while the R_g , R_h , $[\eta]$ values increase the proton mobility decrease. R_g vs. proton mobility graph shows that, HA with 103-112 nm range has highest proton mobility. Between 112-160 nm proton mobility decreases sharply, over 160 nm there is no significant change and has the lowest mobility. In the $[\eta]$ vs. proton mobility, one turning point is obtained in the trend. This turning point is the chain overlap point where the HA chains start to touch and overlap. Chain overlap point is determined as 1233,3 ml/g. Above this viscosity HA chains fill the solvent volume totally and they interact efficiently, as a result proton mobility decreases sharply.

In order to investigate the effect of conformation of HA on the proton mobility, R_g/R_h is calculated and plotted against to proton mobility. The graph showed that HA has highest proton mobility with worm like rod conformation.

In this study, results showed that HA solutions lose their highly viscous, gel like behavior with decreasing dimension of polymer coils in solution. Chains cannot interact effectively and intra- and intermolecular hydrogen bonds get weaker. As a result of absence of strong non-covalent interactions hydrogens can tumble freely and become visible in solution $^1\text{H-NMR}$. Maximum proton mobility of HA is determined in worm like rod conformation with the 103 nm R_g , 46 nm R_h and 581,5 mL/g intrinsic viscosity.

In order to investigate the effect of proton mobility on the calculation of degree of substitution, $^1\text{H-NMR}$ analysis of benzylamine modified HA has been done in D_2O and D_2O with 0,5 M NaOD. DS% is calculated as 23% and 15% for benzylamine modified HA in only D_2O and D_2O with 0,5 M NaOD, respectively. We found that proton mobility affects the calculation of degree of substitution, by using the new method we proved it is possible to obtain correct degree of substitution.

In conclusion, this study obtained direct correlation between proton mobility and polymer dimension. Independent of added reagent or solvent, it is possible to determine the proton mobility of same or similar polymer studied just by measuring the dimension of polymer in solution. The dimension of polymer must be under the chain overlap point in order to perform correct NMR measurement.

HYALÜRONİK ASİT'İN ÇÖZELTİDEKİ KONFORMASYONUNUN PROTON HAREKETLİLİĞİNE ETKİSİ

ÖZET

Hyaluronik asit (HA) tekrarlayan D-glukuronik asit ve N-asetilglukozamin disakkarit birimlerinden oluşan bir biyopolimerdir. Çeşitli kaynaklardan elde edilen HA'ların molekül ağırlığı 10^4 ve 10^7 dalton arasında değişmektedir. Fizyolojik koşullarda sodyum tuzu biçiminde bulunur ve bundan dolayı polimer zincir üzerinde negatif yük taşır, polielektrolit karakter gösterir. Omurgalı hayvanlarda HA dokunun temel bileşenidir ve horoz ibiği, insan göbek bağı, eklem sıvısı ve göz küresinde bulunur. Bunlara ek olarak insan vücudundaki HA'nın neredeyse yarısı deri hücreleri içersinde tespit edilmiştir. Genel olarak HA horoz ibiğinden ekstrakte edilerek üretilmiştir, lakin ekstrakte edilen HA'nın içinde protein safsızlıklarının bulunması biyomedikal ve eczacılık alanlarındaki uygulamalar için büyüyen endişelere neden olmuştur. Bu sebepten dolayı, bakteriyel fermentasyon ile HA üretimi alternatif olarak ortaya çıkmıştır. Son günlerde esas olarak kullanılan üretim yöntemi bakteriyel fermentasyondur.

HA lineer bir polisakkarittir. Yüksek molekül ağırlıklı HA'nın yüksek nem tutma kapasitesi ve viskoelastisitesi, bunlarla beraber toksik özelliğinin olmaması ve vücuda girdiğinde bağışıklık sisteminin antikor üretmemesi gibi özellikler bu polisakkariti benzersiz kılmaktadır. Bu özellikler HA'nın estetik, artoloji (eklem bilimi), oftalmoloji (göz bilimi), onkoloji, yara iyileşmesi gibi çeşitli uygulama alanlarında yer bulmasını sağlamaktadır. Bu uygulama alanları için ham ya da modifiye edilmiş HA kullanılmaktadır. HA'nın bu alanlarda kullanılabilmesi için kimyasal yapısının kesin olarak analiz edilmesi gerekmektedir. İnfrared spektroskopi, ninhidrin analizi ve nükleer manyetik rezonans yapısal analiz için en sık kullanılan yöntemlerdir. Sıralanan yöntemler arasında, kolay numune hazırlanması ve kısa analiz süresinden dolayı hidrojen nükleer manyetik rezonans (^1H -NMR) spektroskopi en çok kullanılan analiz yöntemidir. Fakat, her polielektrolit gibi HA polimer zincirlerinin çok seyreltik polimer çözeltisinde bile birbirleri ile etkileşmesi, geleneksel çözelti ^1H -NMR ile HA'nın analizini oldukça zorlaştırmaktadır. HA polimer zincirlerinin birbiri ile etkileşimi çok seyreltik çözeltinin bile yüksek viskozite göstermesine neden olur, ayrıca proton hareketliliğini etkileyerek ^1H -NMR spektrumunda hangi fonksiyonel gruba ait olduğunun belirlenmesi zor olan geniş piklerin oluşmasına neden olur.

Yüksek viskoziteli sulu HA çözeltileri jel benzeri özellik gösterir. Jeller hareketli ve hareketsiz olmak üzere iki farklı bölgeden oluşur. Geleneksel çözelti ^1H -NMR metodu ile sadece hareketli bölgedeki hidrojenler analiz edilebilir. Polimerin hareketsiz bölgesindeki hidrojenlerin analizi için katı-hal ^1H -NMR metodunun kullanılması gereklidir. HA'nın sulu çözeltisinde, polisakkarite ait hareketsiz bölgenin varlığını göstermek amacıyla katı-hal ^1H -NMR analizi yapılmış ve elde edilen spektrumda HA'nın anomerik, asetil ve şeker iskeletindeki hidrojenler

belirlenmiştir. Böylelikle sulu çözeltideki HA'nın çözelti $^1\text{H-NMR}$ ile gözlenemeyen hareketsiz bölgenin varlığı gösterilmiştir. Polimer çözeltilerinin iyonik gücünü ve pH'ını değiştirerek hareketsiz polimer bölgelerini hareketli hale getirmek mümkündür. Bu çalışmada bu iki parametre değiştirilerek hareketsiz bölgedeki protonların hareketli hale getirilmesi ve bu değişikliğin HA polimerinin konformasyonuna etkisinin incelenmesi, ayrıca konformasyon ile proton hareketliliği arasında bir bağlantı kurulması hedeflenmiştir. HA'nın hareketsiz bölgelerinin hareketli hale getirilerek $^1\text{H-NMR}$ ile görülür olması ve bütün protonların görülür olduğu $^1\text{H-NMR}$ spektrumu kullanılarak modifiye edilmiş HA'nın süstitüsyon derecesinin doğru şekilde hesaplanması amaçlanmıştır.

Çalışmamızda, yüksek molekül ağırlıklı mikrobiyal HA ($1,5 \times 10^6$ g/mol) kullanılmıştır. Analizler için katı HA belirli türde ve konsantrasyonda reaktif içeren çözeltilerde çözünmüştür. Bu çözeltiler şu şekildedir: 0,075 M, 0,125 M, 0,5 M, 1 M, 2 M sodyum klorür (NaCl); 0,071 M, 0,125 M, 0,25 M, 0,5 M, 1 M sodyum hidroksit (NaOH); 0,25 M, 1 M, 4 M üre; 0,5 M sülfürik asit (H_2SO_4) ve 0,5 M hidroklorik asit (HCl). Proton hareketliliğini belirlemek için döteryum oksitli reaktif çözeltileri kullanılarak 4 mg/mL HA çözeltisi hazırlanmıştır, standart olarak disodyumsüksinat hazırlanan çözeltilere eklenmiş, $^1\text{H-NMR}$ ile analiz sonucu elde edilen spektrallardan proton hareketliliği hesaplanmıştır. Her bir reaktifin HA'nın proton hareketliliği üzerinde etkisi incelendiğinde, en fazla hareketlilik NaOH eklenmesiyle sağlanmıştır, ve bunu sırasıyla NaCl, H_2SO_4 , HCl ve üre takip etmektedir. En yüksek proton hareketliliği 0,5 M NaOH içerisinde gözlenmiştir.

HA'nın her bir reaktif çözeltisi içindeki jirasyon (R_g) ve hidrodinamik yarıçapı (R_h) statik ve dinamik ışık saçılması tekniği ile belirlendi. İçsel viskozite ($[\eta]$) ise viskozimetrik ölçüm sonuçlarının excel'de hazırlanan programda kullanılmasıyla hesaplandı. Reaktiflerin R_g , R_h , $[\eta]$ üzerindeki etkisi incelendi. Elde edilen sonuçlara göre artan NaCl, NaOH konsantrasyonu ile R_g değeri küçülmekte, fakat artan üre konsantrasyonu ile tersine R_g değeri artmaktadır. R_g değerinde en fazla azalmaya NaOH reaktifi neden oldu. R_h üzerindeki etki incelendiğinde ise, tüm reaktiflerin artan konsantrasyon ile HA zincirlerinin R_h değerinin azalmasına neden olduğu gözlemlendi. En fazla azalma yine NaOH ile gözlemlendi. Reaktiflerin içsel viskozite üzerindeki etkisi, hidrodinamik yarıçap üzerindeki etkisi ile aynı şekildedir. R_h ve içsel viskozite birbirine bağlı iki parametre olduğundan bu sonuç beklenilmektedir.

Bulunan R_g , R_h , $[\eta]$ değerleri proton hareketliliğine karşı grafiğe geçirildi. Her üç grafik için de benzer bağlantı bulundu, artan R_g , R_h , $[\eta]$ sonucu proton hareketliliği azaldı. R_g 'ye karşı proton hareketliliği grafiğinde, HA 103-112 nm aralığındaki jirasyon yarıçapı ile en yüksek proton hareketliliğini gösterdi. Grafikte 112-160 nm arasında proton hareketliliğinde keskin azalma gözlenirken, 160 nm'nin üzerindeki jirasyon yarı çaplarındaki artış proton hareketliliği üzerinde kayda değer etki göstermedi ve en düşük proton hareketliliği bu bölgede gözlemlendi. Grafikteki veriler göz önüne alındığında 103-112 nm arasında HA zincirlerinin sıkıştırılmış küre konformasyonu, 112-160 nm arasında sıkıştırılmış küre konformasyonunun gittikçe genişlediği ve zincirlerin birbirine ile örtüşmeye başladığı, 160 nm üzerinde ise daha da genişlemiş yapı ile zincirlerin birbirine dolanıp HA çözeltilerinin jel özelliği gösterdiği sonucuna varılmıştır.

$[\eta]$ 'ye karşı proton hareketliliği grafiğinde bir dönüm noktası gözlemlendi. Bu dönüm noktası HA polimer zincirlerinin birbirine değdiği ve üst üste bindiği zincir örtüşme noktasını göstermektedir. Bu grafikten zincir örtüşme noktası 1233,3 ml/g olarak

belirlendi. Zincir örtüşme noktasının üstünde HA zincirleri birbiri ile kuvvetli şekilde etkileşmekte ve bu noktadan sonra artan içsel viskozite ile birlikte proton hareketliliği keskin şekilde azalmaktadır. Grafikten elde edilen bu bilgiler doğrultusunda, zincir örtüşme noktasından daha düşük viskoziteye sahip olan HA çözeltilerinde polimer zincirleri birim çözücü hacminde birbirinden bağımsız olarak hareket edebilmektedir, zincir örtüşme noktası ile eş viskoziteye sahip HA çözeltilerinde polimer zincirleri birim çözücü hacmini tamamen doldurmakta, birbirine değmeye ve üst üste binmeye başlamaktadır. Zincir örtüşme noktasının üstünde ise HA çözeltilerinde polimer zincirleri birbiri içine geçmekte, jel tipi karakter göstermektedir.

Konformasyonu incelemek için her bir reaktif çözeltisindeki HA için ayrı ayrı R_g değerleri R_h 'ya orantılandı. Elde edilen R_g/R_h değerleri proton hareketliliğine karşı grafiğe geçirildi. R_g/R_h değerleri ile HA için 3 farklı konformasyon belirlendi. Bunlar jel veya kümelenmiş sıkıştırılmış küreler, düzensiz sargı ve solucan benzeri zincirdir. HA çözeltileri en yüksek proton hareketliliğini solucan benzeri zincir konformasyonunda gösterdi.

Polimer zincirlerinin boyutlarının küçülmesi ile HA çözeltilerinin yüksek viskoziteli, jel tipi özelliğinin kaybolduğu gözlemlendi. Bu durum polimer zincirleri birbiri ile güçlü biçimde etkileşemediğini ve molekülleri ve moleküller arası hidrojen bağları zayıfladığını gösterdi. HA'nın çözelti içindeki yapısını belirleyen ve yapısal kararlılığı sağlayan hidrojen bağlarının olmaması halinde, polimer üzerindeki hidrojenler çözelti içersinde daha fazla hareket edebilir ve bu da hidrojenlerin çözelti $^1\text{H-NMR}$ analizlerinde görünür olmasını sağlar. Yaptığımız çalışma sonucunda HA'nın maksimum proton hareketliliği solucan benzeri zincir konformasyonunda 103 nm R_g , 46 nm R_h ve 581,5 mL/g içsel viskozite ile 0,5 M NaOH çözeltisi içinde belirlendi.

Proton hareketliliğinin süstitüsyon derecesi (DS) hesabı üzerindeki etkisi araştırıldı. Bu amaçla benzilamin ile modifiye edilmiş HA'nın sadece D_2O ve 0,5 M NaOH içeren D_2O içinde $^1\text{H-NMR}$ analizleri yapıldı. %DS D_2O içersinde %23, 0,5 M NaOH içeren D_2O içinde %15 olacak şekilde hesaplandı. Yapılan deneyler sonucunda proton hareketliliğinin %DS hesaplamalarını büyük ölçüde etkilediği saptandı.

Sonuç olarak, bu çalışmada proton hareketliliği ile polimer boyutu arasında direkt ilişki bulundu. Kullanılan reaktif ve çözücünden bağımsız olarak, HA ve benzer yapıdaki polimerlerin sadece çözelti içersindeki boyutu ölçülerek proton hareketliliği saptanabilir. Doğru NMR ölçümleri için polimer boyutunun zincir örtüşme noktasının altında olması gereklidir.



1. INTRODUCTION

Hyaluronic acid (HA) is polysaccharide made of D-glucuronic acid and N-acetyl glucosamine repeating units. Due to its high capacity for holding water and high viscoelasticity, HA has an outstanding position among biological materials. These properties make it suitable for various medical and pharmaceutical applications such as viscosupplementation therapy for osteoarthritis treatment, oncology, wound healing eye surgery, tissue augmentation, drug conjugates, dermal filling and intradermal micro implants.

It is very common to modify hyaluronic acid to improve its mechanical properties and prolong the duration of HA *in vivo* (Kenne et al., 2013; Khabarov et al., 2014). Furthermore, it is possible to attach the drugs covalently to HA chain and produce polymeric prodrugs.

Nuclear magnetic resonance (NMR) spectroscopy has been considered the utmost advanced and information rich technique for structural analyses of biomolecules, including complex carbohydrates (Pomin, 2013). Nevertheless, in ^1H -NMR spectrum of modified HA broad signals are observed which are very complicated to assign and cause error in degree of substitution calculations. This effect could be explained by that HA in solution has not full proton mobility. The protons of HA used as reference in the NMR have not the same mobility as the protons of the grafted molecule. In order to perform a correct degree of substitution determination in modified products, all the protons in the molecule of HA should have full proton mobility.

1.1 Purpose of Thesis

The aim of this study is investigation into the influence of HA conformation in solution on the proton mobility and obtain highest proton mobility of HA for correct degree of substitution calculation. For this purpose, HA solutions of different ionic

strength and pH were investigated by ^1H -NMR spectroscopy, viscosimetric and light scattering measurements.

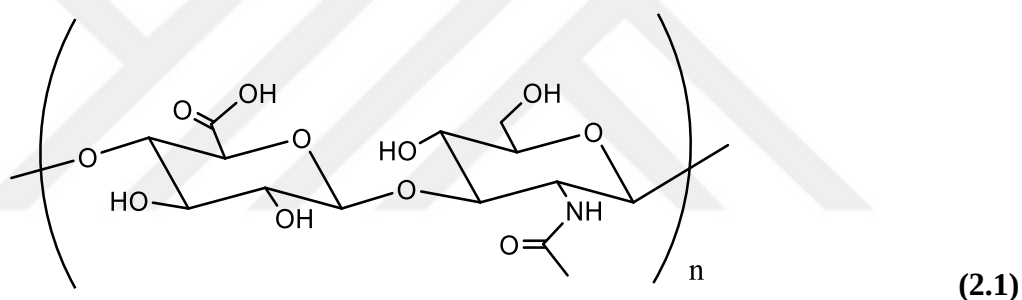


2. THEORY

2.1 Hyaluronic Acid

2.1.1 Structure and properties of hyaluronic acid

Hyaluronic acid (HA) is a linear polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked by $\beta(1,4)$ and $\beta(1,3)$ glucosidic bonds shown as formula (2.1) (Schanté et al., 2011). One carboxylate group per disaccharide unit provides polyelectrolyte behavior to polysaccharide (Cowman and Matsuoka, 2005). All bulky groups (hydroxyl, carboxyl and acetamide) and a glycosidic bond orientated equatorial which is sterically more favourable position. Hydrogen atoms tend to be in axial position, therefore part of the molecule is hydrophobic. On the other hand the carboxyl, hydroxyl and acetamide groups supply hydrophilic properties to the polysaccharide molecule.



Hyaluronan's facility to interact with different chemical compounds and turn into different conformations mostly relies on the presence of polar and non-polar fractions in the macromolecule's structure (Khabarov et al., 2014).

Primary structure of HA is characterized by identifying all composing monosaccharide residues, the anomeric configurations and the positions of any substituents. In early studies, testicular and bacterial hyaluronidases used for hydrolyze just the N-acetylglucosamidic bonds of HA. The degradation products of these two enzymes showed an equal molar of glucuronic acid and N-acetyl glucosamine. These results affirmed that HA has a linear structure with alternating sequence of two sugar units.

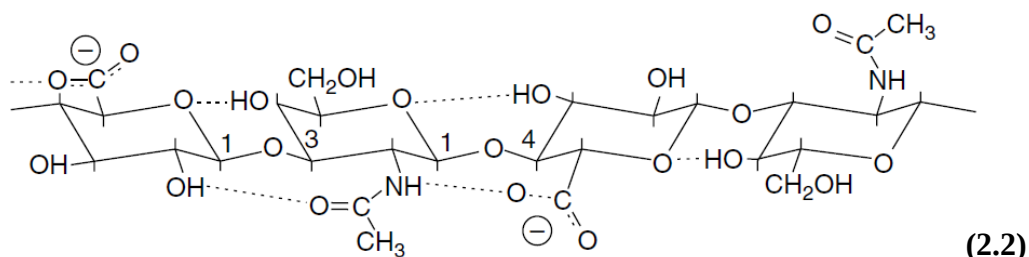
The secondary structure essentially depends on the spatial relationship between neighbouring functional groups. The effect of spatial relation can be noticed in the rigidity of the polysaccharide chain. The degree of rigidity mainly relies on the limit

on the rotation of the glycosidic bonds caused by the geometry of sugar rings, and for HA especially by the bulky N-acetyl group adjacent to glycosidic bond (Kuo, 2005).

The researches on conformation analysis of hyaluronan in aqueous solutions has been conducted by different methods such as x-ray, laser light scattering and viscosimetry. In the results, various conformations of hyaluronan were obtained depending on ion surrounding, concentration, pH, etc. Guss et al. showed that each sodium hyaluronate polysaccharide chain is left-handed 4 fold helix of disaccharide unit. Using viscosimetry and sedimentation coefficients in The Haug Triangle, it was shown that the gross conformation of HA in aqueous solution is random coil (Hokputsa et al., 2003). In another publication, helical structures have been determined from strontium and calcium salts of HA by using x-ray diffraction data. Study presented that neighboring antiparallel chains are binded together through $\text{COO}^- \cdots \text{Ca}^{+2} \cdots \text{OOC}^-$ bridges and the co-ordination of each Ca^{+2} ion is supported by three pairs of water molecules (Winter and Arnott, 1977). Light scattering studies on hyaluronic acid extracted from umbilical cord showed that polydisaccharide has kind of rigid coil structure which does not change its molecular property in the pH range 1,8 to 7,0 (Laurent and Gergely, 1955). In another research, it has been found by using low angle X-ray and viscosimetry measurements that HA has the worm-like coil conformation with a persistence length approximately 4 nm both in acid and salt solutions (Cleland, 1977).

HA macromolecule is able to band and form left-oriented single and double helixes or multi-strand plane structures. Thus its primary and secondary structure, ordered structure such as helix is possible for HA. In aqueous solution of HA, interchain and intrachain hydrogen bonds are formed on the macromolecule and stabilize the structure. Between the oxygen of the carboxyl group and the hydrogen of the acetyl amide group, hydrogen bonds are created directly or through water molecule which works as a bridge. In addition; other hydrogen bonds are formed between hydrogen of the hydroxyl group that orientates to equatorial plane and oxygen atom of glycosidic bond. As a result, with formed hydrogen bonds sugar units (disaccharides) are orientated to opposite directions, up and down as formula 2.2. Hydrophobic regions on neighbouring molecules are close, subsequently hydrophobic bonding between them can occur. While electrostatic repulsion between carboxyl groups promotes the dissociation of aggregates, hydrogen and hydrophobic bonds lead

macromolecule to aggregate; equilibrium amongst these two contrary effects on macromolecule cause helix-coil conformation transition (Khabarov et al., 2014; Rinaudo, 2006).



HA and its salts with magnesium ion, ammonium ion and alkali metals are good soluble in water. On the other hand, hyaluronan salts with cations of two and more valence are practically insoluble in water; these cations cause intermolecular cross link which forms stable gel structure with high water content. With increase of polymer concentration the intermolecular network in HA solutions is formed but in the diluted aqueous solutions HA behaves like an independent molecule. In high concentrations, the web-like structure is observed. Concentration and molecular weight of polysaccharide are significant factors for the formation of a network structure. Study with electron microscopy shows that high molecular weight HA forms network structure in solution even at very low concentrations. In addition, low molecular weight HA forms a network structure at low concentrations in aqueous solutions. Nonetheless, the network has an island-type structure, where some areas are split by the important distance (Khabarov et al., 2014).

Lowering pH has an effect of decrease on the dimension of HA by the transformation of a negative charged polyelectrolyte to a neutral polymer. As a result of neutral conditions, there is no electrostatic repulsion between the HA polymer chains. The chains can come closer and aggregates (Figure 2.1).

Furthermore, increasing pH disrupts inter- and intramolecular hydrogen bonds, turns hydroxyl groups on the HA chain into anionic form (-O^-) and leads to intramolecular cleavage of glycosidic bonds. However, still HA maintains its polyelectrolyte character (Ghosh et al., 1993).

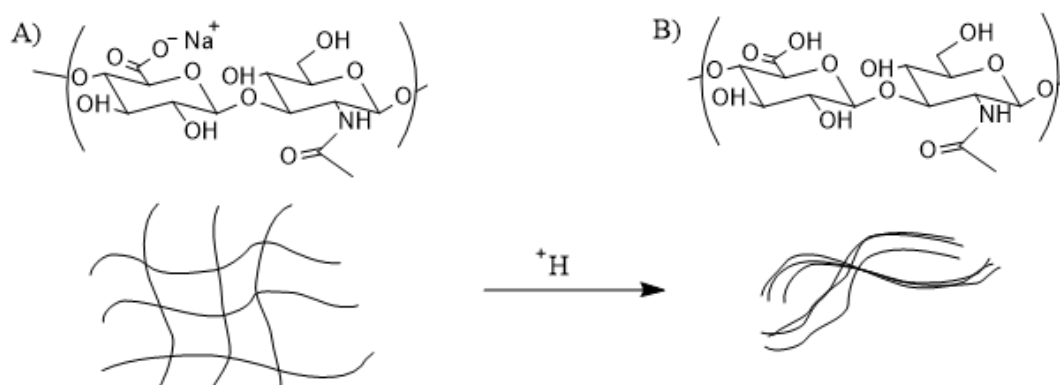


Figure 2.1 : Schemetic presentation of hyaluronic acid in solution A) sodium salt form B) neutral form.

2.1.2 Source and isolation

HA is essential component of tissues in the vertebrate organism. The primary structure of HA is same for all species, but the difference is that the molecular weight and polydispersity index of polymer vary through species. As it can be seen in Table 2.1, highest amount of HA is found in the rooster comb and then human umbilical cord and synovial fluid follows. In addition, almost half of the human body's HA occurs intracellularly in the skin. It doesn't only supply a matrix where cells are embedded, but also immobilizes water in tissue, influence cell proliferation, differentiation and tissue repair. Recently, it has been known that HA is a metabolically highly active biopolymer. It's halftime in epidermis and derma is up to 1-2 days and only 2-5 minutes in blood stream.

Table 2.1 : Amount of HA in different animal tissues (Kogan et al., 2007).

Tissue or body fluid	Concentration (µg/mL)
Rooster comb	7500
Human umbilical cord	4100
Human synovial fluid	1400-3600
Bovine nasal cartilage	1200
Human vitreous body	140-340
Human dermis	200-500
Human epidermis	100
Rabbit brain	65
Human urine	0,1-0,3
Human serum	0,01-0,1

Hyaluronic acid can be extracted from tissue of vertebrate organism; for example rooster comb, shark skin, bovine eye-balls. The procedure consists of steps as tissue homogenization, extraction, purification and preparation of final commercial HA

product. The extract from the animal contains the impurities such as proteins, peptides, lipids, nucleic acids, mucopolysaccharides and low molecular weight precursors. Therefore, several purification procedures have to be applied in order to obtain a pure compound such as protease digestion, HA ion-pair precipitation (e.g with cetylpyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and/or lyophilization.

HA can also be prepared from microbial fermentation, with this production way it is possible to get HA with molar mass in range of several million dalton (MDa). The disadvantage of this method is that due to possible mutation of bacterial strains, various toxins, pyrogens, immunogens might be exist in product.

As a consequence of identifying the enzymes responsible for HA synthesis, some bacteria strains genetically modified to carry the gene hyaluronan synthase. Hyaluronan synthase encodes the enzyme HA synthase which produces the HA. This modified strain is easily cultivatable in a large scale and doesn't produce exo- or endotoxins. The production of HA from the cultural media consist of steps following, the removal of the microorganism, removal of low molecular weight substance by ultrafiltration, precipitation with organic solvent and purification of the final product by using the methods mentioned before (Khabarov et al., 2014; Kogan et al., 2007).

2.1.3 Application of hyaluronic acid

HA is high molecular weight, linear polydisaccharide. It has unique properties like retaining large amount of water and forming highly viscous solutions. These two properties provides a wide range of application areas such as aesthetic medicine, arthology, ophthalmology, oncology and wound healing (Khabarov et al., 2014; Sagle et al., 2009).

Osteoarthritis is most common type of arthritis, affecting millions of people worldwide. This disease is caused by the reducing glucosamine synthesise ability of cartilage tissue chondrocytes. The healthy knee contains approximately 2 mL of syndovial liquid, the HA in it is in the range of 2,5-4 mg/mL. Patients with osteoarthritis have 2-3 times lower synthesis rate and concentration of HA in syndovial liquid compared to healthy people. In this patients the molecular weight of HA decreases because of accrelated hydrolysis, as a result the viscoelactic properties of the syndovial liquid is reduced. Lower viscosity and concentration of HA cause

friction on the surfaces of the cartilage and lead to destruction of cartilage and bone tissue. For the treatment of osteoarthritis, the products of high molecular weight or cross-linked HA products are injected directly to the patient knee. There are many data that proves the effects of the intra-joint injection of HA on reduction of pain and improving functional activity of joints. Furthermore, it was confirmed that application of HA support to recover the properties of synovial liquid, activate anabolic processes and reduce catabolic processes in the cartilage tissue (Khabarov et al., 2014; Sagle et al., 2009).

The amount of hyaluronic acid in the human skin changes depending on season, in summer level of HA is low compare to the winter. The reason is that UV radiation from sun degrades HA at higher rate. However, the most significant effect is the age-related decrease in HA concentration. According to a study with different age group of patients showed that the concentration of HA in derma rapidly decreases from the age of 60 years. Hence, intradermal injection of unmodified HA contributes to recovery of the physiological medium and normalizing the metabolic processes in derma. The molecular weight has the most significant effect on the mechanism of physiological regulation. The low-molecular weight fraction of HA promotes inflammatory action whereas high molecular weight inhibits. Therefore, for injections molecular weight of HA should be strongly controlled to contain only narrow fractions with defined molecular weight (Khabarov et al., 2014).

HA is used also in skin care creams and lotions because it helps to moisturize the skin, prevent the dryness and loss of elasticity which occurs in aging skin.

Hyaluronan products are commonly used in the general and local therapy of eye diseases. Dry corneas are treated by eye drops (artificial tears) containing HA in the formulation. In order to protect eye's internal tissues from mechanical hazards in eye surgery procedures, a solution is used which contains high molecular weight HA. Moreover, the vitreous body of eye mainly consists of HA. Viscoelastic properties of HA provides the vitreous body to have a stable intraocular pressure and prevents detachment of retina and pigment epithelium. Several formulations of HA solutions with aminoacids, antioxidants and different compounds has been developed and they can be used for eyeball irrigation, or injection into eye's anterior chamber during cataract surgery.

HA is used as a therapeutic in the field of oncology. There are various mechanisms of HA acts on the tumor cells. One of them is, high molecular weight HA can bind to the receptor on the cell membranes of tumor cells and inhibit the growth of cancer and metastasis, because with the aid of these receptors cancer cells invade and form metastasis. Another mechanism of high molecular weight HA is to inhibit tumor vascularization, which leads to the slow growth and metastasis of tumours. It is really interesting that contrary to high molecular weight HA, which inhibits tumor vascularization, the low molecular weight HA (below 30000Da) induces it. Tumor cells secrete extracellular hyaluronidase to hydrolyze the hyaluronan in extracellular matrix for invasion. Hence, the formulation of high molecular weight HA in combination with potent hyaluronidase inhibitors can be effective on cancer treatment (Khabarov et al., 2014).

HA can be used in combination with other drugs to quicken the healing of burns, trophic ulcers and surgical procedures. HA is used for treatment of the stomach and duodenum ulcers. The anti-ulcer action of HA is related on being able to inhibit H₂ histamine receptors and activity of trypsin. Low molecular weight fractions of HA can penetrate through the skin epidermal barrier, thus it can be used for transporting bioactive compounds. In addition, the healing of skin wounds is directly linked to increasing of HA during the first three days and decrease to the initial level seven day after the injury. That is why HA is used as a primary wound healing product or in combination with different reagents (Khabarov et al., 2014; Sagle et al., 2009).

2.1.4 Modification of hyaluronic acid

Two different chemical methods can be used for HA modification: cross-linking or conjugation. A compound is grafted onto one HA chain only by single bond for conjugation method, on the other hand different HA chains are linked by two or more bonds for cross-linking (Figure 2.2).

The chemical modification of HA can be performed on two functional groups, the carboxylic acid and hydroxyl group. Modification of HA has been done in order to develop HA derivatives with appropriate characteristics according to applying areas. Some areas low substitution or crosslinking degrees is desired, whereas for other areas, high degrees of substitution or crosslinking is required. Thus it is really

significant to know the exact degree of substitution and crosslinking of HA derivatives.

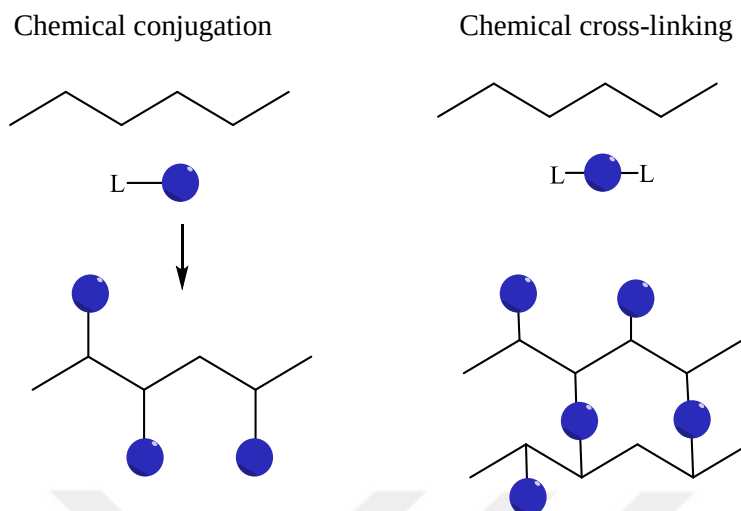


Figure 2.2 : Schemetic presentation of chemical conjugation and cross-linking.

Modification is used to improve HA's physicochemical and mechanical properties. For instance, due to short life of HA solutions in vivo, most commercial HA products for articular injections and dermal fillings are crosslinked to obtain non-soluble hydrogels with longer residence times and mechanical properties. Native HA has intra-articular residence time for some hours. When native HA chemically cross-linked this time increases up to some weeks. Injection related infection is increased by a high number of administrated injections. In order to avoid this adverse effect, low number of injections should be applied to patients. Cross-linked HA with longer residence time can overcome adverse effects caused by multiple injections. In addition by cross-linking it is possible to incerase the viscosity and density of HA while biocompability remains same with native form (Ågerup et al., 2005).

Furthermore, by conjugation technique it is possible to covalently bind a drug to HA chain. When the covalent bond between HA and binded drug is broken down in organism, the drug is released to the specific target area.

Modified HA's physicochemical and mechanical properties alter extremely depending on degree of substitution (DS%). DS% is the proportion of the modifying agents that are substituted to the total number of HA disaccarides. Calculating the DS% of modified HA is important in order to understand different properties of modified polymers and the effects of diverse manufacturing processes used.

Detailed structural analysis of modified HA is really hard to perform due to the high viscous character of polymer solution and the complex cross-linked network of HA hydrogels. Kenne et al developed one method to analyze accurately DS% of the modified HA. This method consists of two steps; first degradation of HA with enzyme, second analyzing the digest products by size exclusion chromatography combined with electrospray ionization mass spectrometry (Kenne et al., 2013). In another study, modified HA is degraded with enzyme and all fractions collected by size exclusion chromatography. Oligomers are characterized by NMR and LC-MS method (Wende et al., 2016). However these two methods are expensive and time consuming. In this study a method is developed to determine the DS% accurately, which has easy sample preparation, highly reproducible and quick.

2.2 Characterization Techniques for Conformational Analysis

2.2.1 Rheology

The viscosity is the ratio of the shear stress δ to the shear rate γ :

$$\eta = \frac{\delta}{\gamma} \quad (2.3)$$

The shear stress δ is the force F [N] applied on the surface A [m²] which is parallel to the direction of the flow (equation 2.4):

$$\delta = \frac{F}{A} \quad (2.4)$$

The shear rate is the ratio of velocity difference Δv [m/s] between two fluid layers to the distance Δh (equation 2.5). The unit of shear rate is [s⁻¹].

$$\gamma = \frac{\Delta v}{\Delta h} \quad (2.5)$$

The ratio of the viscosity η of solution to the viscosity of the pure solvent η_s is the relative viscosity and called η_r :

$$\eta_r = \frac{\eta}{\eta_s} \quad (2.6)$$

When the viscosity accepted as the sum of the solvent viscosity η_s and the viscosity of the dissolved polymer η_p :

$$\eta = \eta_p + \eta_s \quad (2.7)$$

On that occasion, the ratio of the dissolved polymer viscosity η_p to the solvent viscosity η_s is the specific viscosity η_{sp} of the polymer in solvent, shown as (equation 2.8):

$$\eta_{sp} = \frac{\eta_p}{\eta_s} = \frac{\eta - \eta_s}{\eta_s} = \eta_r - 1 \quad (2.8)$$

The specific viscosity still depends on concentration. In order to discard the interactions among polymer chains and determine only the interaction between the polymer and solvent, the reduced viscosity is calculated as shown in equation 2.9.

$$\eta_{red} = \frac{\eta_{sp}}{c} \quad (2.9)$$

This reduced viscosity only depends on polymers density in solution, but it is not completely independent from the concentration. One possible way to compare viscosity measurements is to use intrinsic viscosity. Intrinsic viscosity $[\eta]$ can be defined as a measure of the hydrodynamic volume of HA chain in the solution. The reduced viscosity extrapolated to $c \rightarrow 0$ (Kulicke and Clasen, 2013). As shown in equation (2.10).

$$[\eta] = \lim_{c \rightarrow 0} \eta_{red} \quad (2.10)$$

When η_{red} plotted against concentration a relation is observed (Figure 2.3). It shows that although working with diluted solutions there is still intermolecular interactions between polymer chains. These interactions can be investigated by Huggins equation shown in equation (2.11).

$$\eta_{red} = \frac{\eta_{sp}}{c} = [\eta] + K_H \cdot [\eta]^2 \cdot c \quad (2.11)$$

The Huggins constant K_H is a measure for these intermolecular interactions. In Figure 2.3, the slope of the reduced viscosity is equal to $K_H \times [\eta]^2$ related to the Huggins equation. It describes solvent quality that presents expansion of polymer coil and interaction between polymer chains in solution. For instance, the intrinsic viscosity of polyacrylamide (PAAm) increases with the solvent quality also the slope of the curves in the linear region.

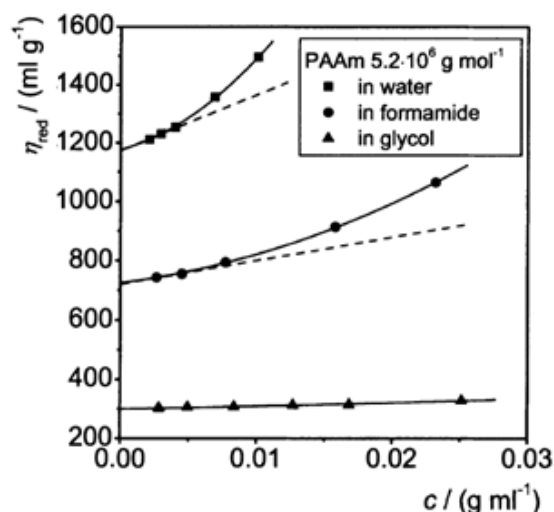


Figure 2.3 : Reduced viscosity as a function of c for polyacrylamide (PAAm) in the solvents water, formamide, ethylenglycol (Kulicke and Clasen, 2013).

The dependence of $[\eta]$ on molecular weight for general conformation types is well represented by the Mark-Houwink-Sakurada relation (equation 2.12):

$$[\eta] = K \times M^{\alpha} \quad (2.12)$$

M is molecular weight, K and α are parameters depends on polymer-solvent system. α parameter gives information about the structure of the polymer in the solvent. Figure 2.4 shows the polymer structure in solution depending on the α value.

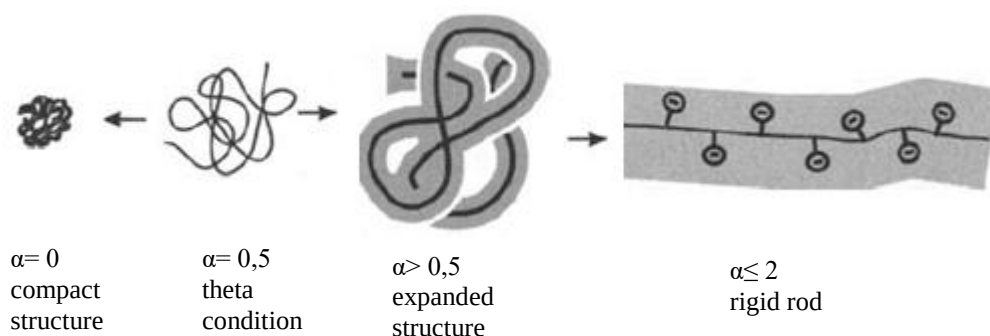


Figure 2.4 : Schematic representation of different stages of the solution conformation of dissolved polymer in solution (Kulicke and Clasen, 2013).

When $\alpha=0$ the structure is aggregated compact sphere or hyperbranched compact. For $\alpha=0,5$ polymer is in theta condition in poor solvent, above this value polymer has expanded conformation, at $\alpha=2$ polymer has semiflexible or rigid rod structure (Kulicke and Clasen, 2013).

Viscosity of newtonian fluids remains constant as the shear rate changes. Water, air and thin motor oil are examples of newtonian fluids. On the other hand, non-newtonian fluids present different behaviour; viscosity of the fluids changes as share rate is differed. There are several types of non-newtonian fluids, pseudoplastics and dilatant are two type of them. Pseudoplastics show a decreasing viscosity with increasing shear rate. This kind of flow behaviour is called as shear-thinning. Opposite to pseudoplastics, dilatant fluids demonstrate increasing viscosity with increasing shear rate and this flow behaviour is called as shear-thickening. Figure 2.5 shows the typical viscosity-share rate plot of Newtonian, shear-thinning and shear-thickening fluids (Wilkes, 1981).

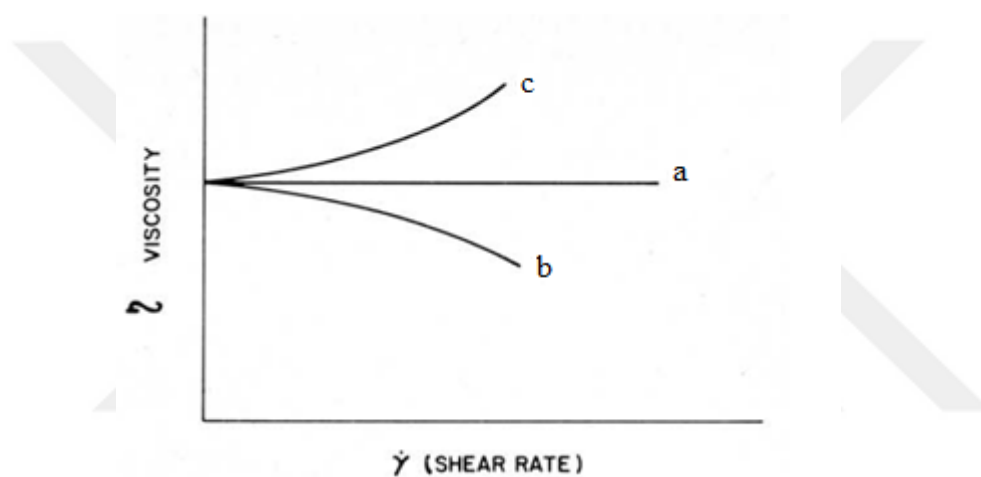


Figure 2.5 : Viscosity shear rate plots for Newtonian (a), shear-thinning (b), shear-thickening (c) fluids (Wilkes, 1981).

In flow experiments, a critical share rate (γ_c) is a point after that the characteristic time applied by shear rate becomes lower than the relaxation time of the solution (Relaxation time is the time associated with large scale motion or change in the structure of polymer in solution). Critical shear rate depends on molecular weight, polymer concentration and rigidity of the chains; when these parameters increase critical shear rate decreases.

Critical concentration c^* , is the polymer concentration where solution is completely filled with polymer coils and the coils start to interact with neighbour chains as shown in Figure 2.6.

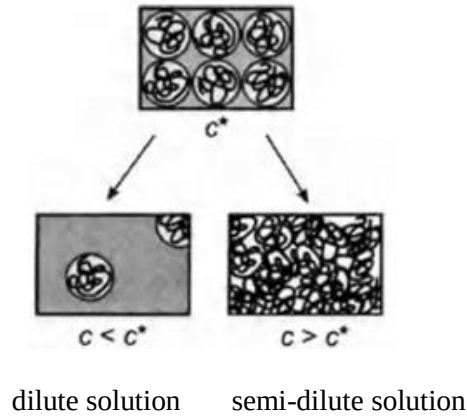


Figure 2.6 : Schematic presentation of critical concentration in polymer solution (Kulicke and Clasen, 2013).

The critical concentration is the boundary between the dilute and semidilute concentrated solutions. With transition from dilute to semidilute region, flow properties of a polymer solution changes seriously. At concentrations above c^* the flow behaviour is controlled by the interactions of polymer coils, when below c^* essentially polymer-solvent interaction regulate the flow properties. The critical concentration of viscosimetry $c_{[\eta]}^*$ is calculated by:

$$c_{[\eta]}^* = \frac{m_{polymer}}{V_{solution}} = \frac{2,5}{[\eta]} \quad (2.13)$$

The critical concentration $c_{[\eta]}^*$ is proportional to the reciprocal intrinsic viscosity. The factor 2,5 accepts that the polymer coils act like hard spheres in solution. For a correct determination of intrinsic viscosity there must be no interaction between the polymer chains in solution, for this reason it is necessary to perform measurements in dilute solutions at concentration clearly below c^* (Kulicke and Clasen, 2013).

2.2.1.1 Structural analysis with rheology

Rheology provides precious tools to explore and understand the properties of polymer systems. The rheological behavior of polymer systems displays the underlying structure of the systems. Viscosity of polymer solutions is directly related to fundamental molecular properties such as molecular conformation, molecular weight, molecular weight distribution, intramolecular interactions and intermolecular interactions.

Intrinsic viscosity is a measure of the effective specific volume (in units of mL/g) of a macromolecule in solution. Its dependency on the length of homologous polymer chains provide a sensitive measure for the gross conformation of macromolecule (Tanford et al., 1967). Determination of intrinsic viscosity as a function of temperature, pH, salt concentration, etc., is commonly used to follow conformational changes of macromolecules. A change in conformation is usually shown by a more or less abrupt change of the viscometric quantity. This method can be used alone but often in association with polarimetric, spectrometric or potentiometric measurements (Lefebvre and Doublier, 2005).

Tsaih and Chen (1997) studied the effects of molecular weight and urea on the magnitude of the Mark-Houwink-Sakurada exponent α of chitosan molecules in dilute solution. They calculated the α values for high molecular weight (HM_w) and low molecular weight (LM_w) chitosan in solution with different ionic strength. It was found that HM_w chitosan has random coil conformation whereas LM_w chitosan has rod shape. Furthermore, in 4 M urea solution hydrogen bonds are destroyed and chitosan conformation becomes extended independent of molecular weight (Tsaih and Chen, 1997).

In another study, hydroxypropyl cellulose (HPC)-surfactant interaction was investigated by rheology and cloud point determination. It was found that modifying HPC either by increasing hydrophobic character or charge density via charged surfactant absorption may result in a relative viscosity increase and an increase in shear-thinning character. They reached the conclusion that this is because of an increase in the “three-dimensional network structure” (Drummond et al., 1992).

Conformation of proteins in solution was studied by Tanford et al. In this study, measurements have been done for determination of intrinsic viscosities and sedimentation coefficients of protein-polypeptide chains in concentrated guanidine hydrochloride, in presence of β -mercaptoethanol which ruptures disulfide bonds. The results showed that both properties are absolutely as predicted for randomly coiled linear polymer chains (Tanford et al., 1967).

Rheological properties of HA are studied in literature by various research groups. Dilute solution rheological behavior of HA in 0,15 M NaCl at 25°C is shown in Figure 2.7 (Cowman and Matsuoka, 2005). For the relationship between $\log[\eta]$ and

$\log M$, the data shows two distinct linear regions due to the different conformation of the molecule (low M_w rod like, high M_w more extended coil-worm like). The low molecular weight region corresponding to the “free-draining chain conformation”. The high molecular region is corresponding to the “non free-draining ball like conformation”. The Figure 2.7 shows a relation between the intrinsic viscosity and molecular weight. While the molecular weight increases, a change from short extended chains to longer chains which forms coiled structures is observed.

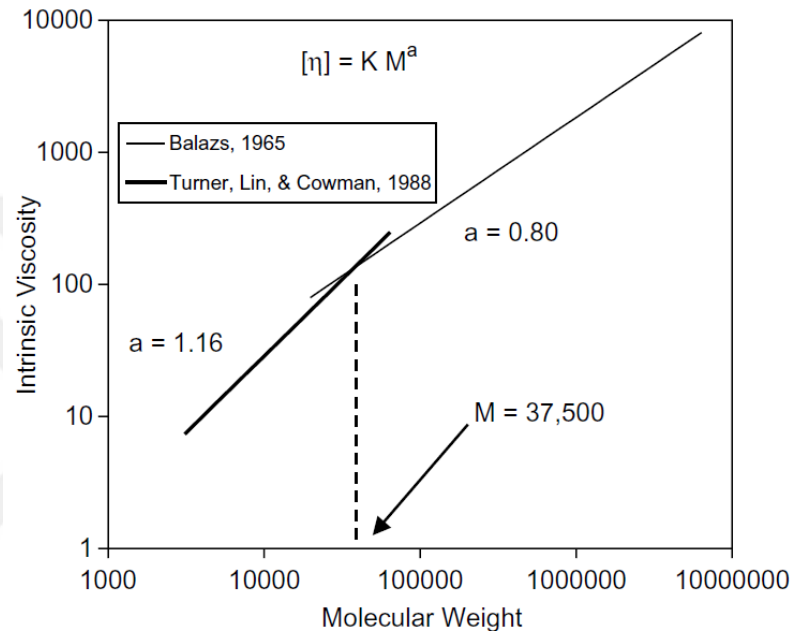


Figure 2.7 : Experimental dependence of intrinsic viscosity on molecular weight of HA in 0,15M NaCl solution (Cowman and Matsuoka, 2005).

In Figure 2.8, some rheological properties of HA are presented. Curves show zero-shear viscosity ($\eta_{sp,0}$), critical concentration (γ_c) after which viscosity becomes shear dependent and slope of the curve (s) in the shear dependent viscosity region which shows the pseudoelastic behaviour of hyaluronate (Eric Fouissac et al., 1993).

Addition of alkali changes the population of conformers, probably due to disrupting the interresidue hydrogen bonds. In alkali conditions, hydroxyl groups of HA become ionized and structure is contracted. As a result, viscosity gets dramatically reduced. This effect is readily reversible by lowering pH (Cowman and Matsuoka, 2005).

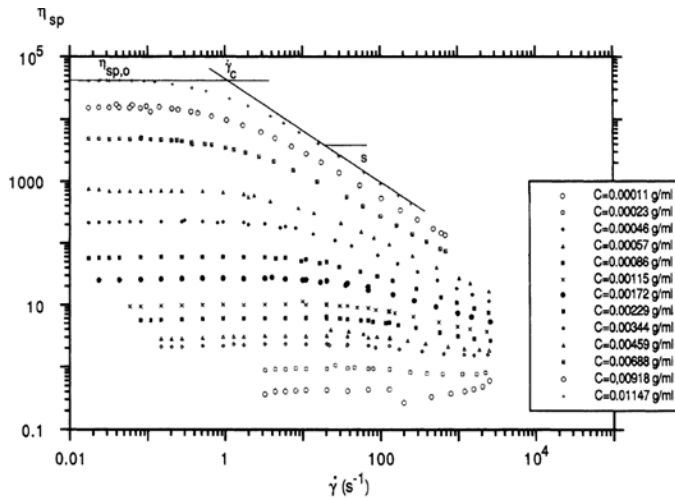


Figure 2.8 : Influence of the shear rate on the specific viscosity of 22×10^7 bacterial HA at different concentrations in 0,1 M NaCl 25°C (Kulicke and Clasen, 2013).

In lower pH acid protonates carboxyl groups of HA, decreases intramolecular electrostatic repulsion and reduces the intrinsic viscosity in the absence of added salt. Furthermore, low ionic strength increases both intra- and intermolecular electrostatic repulsion and the measured intrinsic viscosity (Cowman and Matsuoka, 2005).

2.2.2 Static and Dynamic Light Scattering

Light scattering has been widely used for characterizing polymer chains in a solution. With this method, weight-average molecular weight (M_w), radius of gyration (R_g), hydrodynamic radius (R_h), and second virial coefficient (A_2) can be calculated. Furthermore, it is possible to determine the shape of the polymer whether it is spherical, random-coiled, or rod-like in solution.

Figure 2.9 presents a sample structure of light scattering measurement system. As seen from Figure 2.9, cylindrical test tube contains polymer solution immersed in a glass vat filled with index matching liquid. Index matching liquid has refractive index close to that of glass vat in order to avoid refraction of beam caused by passing through two different mediums. Incident beam enters to the index-matching liquid and propagates through the polymer solution. Nearly all incident beam photons move straightforward into the system and form strong forward scattered beam. Small portion of photons are scattered in all directions by the molecules in incident beam path. A photo detector placed horizontally at an θ angle to the beam path detects the scattered photons.

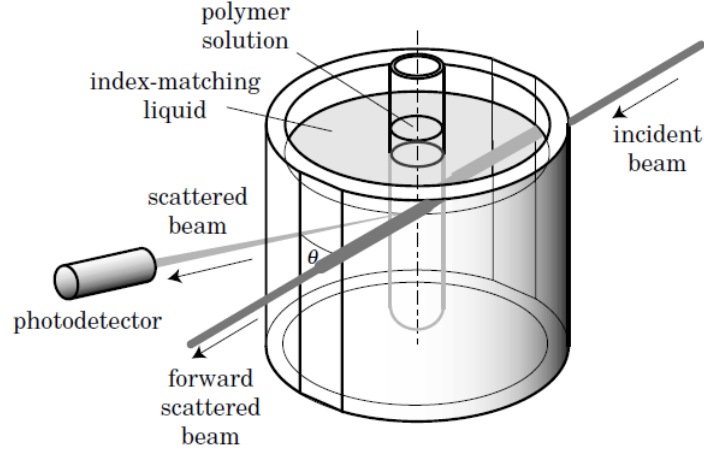


Figure 2.9 : Schematic of the geometry around a sample cell in a light-scattering measurement system (Iwao (2002)).

Radius of gyration (R_g), is used for measuring the chain dimensions. It is defined as the mean square of the distance between beads and the center of mass (Iwao, 2002). R_g is measured by static light scattering (SLS). SLS uses equation 2.14 depends on weight concentration of solutions (not molar) and refractive index increment of solute/solvent system.

$$\frac{K c}{R_\theta} = \left(\frac{1}{M} + 2A_2 c \right) \left[1 + \frac{16\pi^2}{3 \left(\frac{\lambda_0}{n} \right)^2} R_g \sin^2 \frac{\theta}{2} \right] \quad (2.14)$$

In this equation K is constant, c is the concentration of polymer solution, R_θ Rayleigh ratio, M_w molecular weight, $P(\theta)$ particle form factor, A_2 second virial coefficient, λ_0 the wavelength of the incoming radiation, n is refractive index. $P(\theta)$ has the value 1 for $\theta = 0^\circ$. For larger angles $P(\theta)$ is always smaller than 1. Three characteristics of equation 2.14 are used for calculations. For $\theta=0$ and $c=0$ we have equation 2.15:

$$\frac{K c}{R_\theta} = \frac{1}{M} \quad (2.15)$$

For $c=0$ equation 2.14 leads to equation 2.16:

$$\frac{K c}{R_\theta} = \frac{1}{M} + \frac{16\pi^2}{3 \left(\frac{\lambda_0}{n} \right)^2} R_g \sin^2 \frac{\theta}{2} \quad (2.16)$$

For $\theta = 0$ equation 2.14 leads to equation 2.17:

$$\frac{Kc}{R_\theta} = \frac{1}{M} + 2A_2c \quad (2.17)$$

Measuring intensity of scattered photons at zero angle is practically not possible because the laser beam would shine directly into the detector. In this situation, it is impossible to separate scattered photons from un-scattered incident beam.

In order to determine the R_g and M_w of polymer in solution accurately, scattered light intensity is measured at many different angles and extrapolate to zero angle. Moreover, several concentrations of sample are measured in order to be able to extrapolate zero concentration for each scattering angle. It is not practical to take one measurement at very low sample concentration because the scattering would be very low to detect. On the other hand, for very high sample concentrations the virial term in the equation ($2A_2c$) would not be small enough to discard (Url-1). Consequently, all measurements must be done in optimal sample concentrations.

The value of A_2 is a measure of non-ideality of the solution or a measure of interaction forces between dissolved particles: Negative A_2 value shows that the solvent quality is poor for the polymer, polymer-solvent interactions become inconvenient and the polymer chains contract. As a result, polymer conformation in solution changes in order to minimize the polymer-solvent contact and maximize the contacts between polymer chains. The intrachain and interchain attraction becomes stronger, leads to formation of aggregates (Iwao (2002)).

In Figure 2.10 shows one typical zimm plot. The $\frac{Kc}{R}$ values are plotted against $q^2 + kc$, k is arbitrary constant in order to make kc same magnitude as q^2 . In this graph, horizontal lines represent the five different concentrations measured and vertical lines represent the different angles.

The lines with constant concentrations extrapolated to the value where $\theta = 0$ and contrarily the lines of constant angles are extrapolated to $c = 0$. The $c = 0$ and $\theta = 0$ lines intersect at the axis with value of $\frac{1}{M}$. The slope of $\theta = 0$ line is equal to $2A_2$ (Url-2). As shown in Figure 2.10, R_g is calculated from the slope of $c = 0$ line by using calculated M value in the equation 2.16.

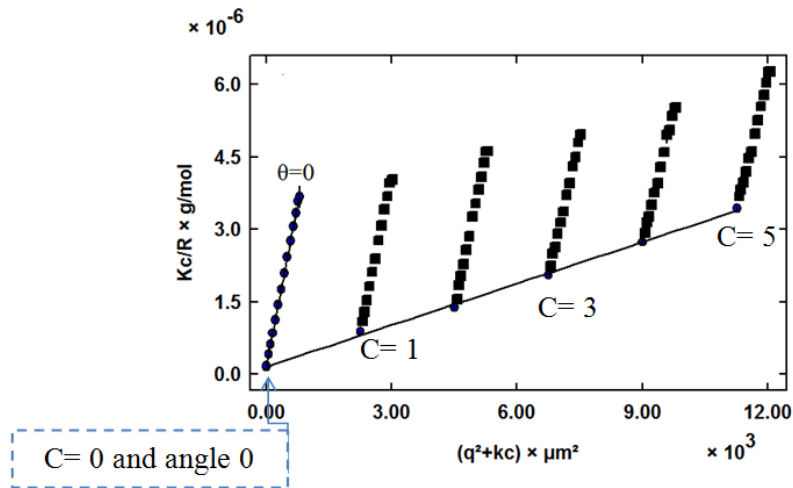


Figure 2.10 : Typical zimm plot results.

Dynamic light scattering (DLS) is commonly used method for analyzing the diffusion related to the Brownian motion of the scattering particles in solution (Koetz and Kosmella, 2007).

Temporal concentration fluctuation causes a change with time in the intensity of scattered light detected at a certain scattering angle. This change reflects the Brownian motion of the scattering particles. For DLS measurements neither the weight concentration nor the refractive index increment need to be known. For SLS experiments, the average scattered intensity is measured. On the other hand, for DLS measurements, the detailed analysis of the fluctuating intensity is significant (Schärftl (2007).

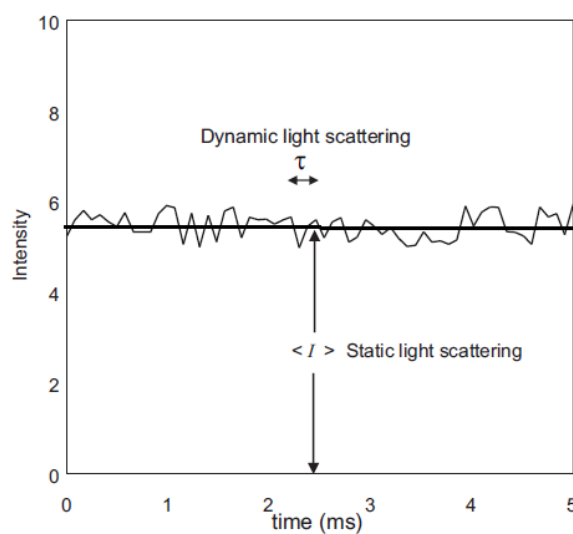


Figure 2.11 : Light scattering intensity of macromolecule vs. time (Url-1).

As shown in Figure 2.11, average intensity of scattered light used for M_w and R_g calculation by SLS, whereas fluctuations in intensity reflect the diffusion coefficient of the molecules.

Diffusion coefficient obtained by DLS measurement is used for calculation of hydrodynamic radius (R_h) by the Stoke's Einstein approximation for the behaviour of a sphere (equation 2.18), where K_B is the Boltzmann's constant, T the temperature and η_s viscosity of the solvent. We can consider R_h as a quantity to characterize dimension of the molecule.

$$R_h = \frac{K_B T}{6\pi\eta_s D} \quad (2.18)$$

DLS/SLS can be combined to obtain the morphology of polymers in solution. For this aim ρ -ratio is used and calculated as :

$$\rho = \frac{R_g}{R_h} \quad (2.19)$$

Theoretically calculated ρ -ratio values of most significant topologies are shown in the Table 2.2:

Table 2.2 : ρ -ratio for the typical paticle morphologies.

Topology	ρ -ratio
Homogeneous sphere	0,775
Hollow sphere	1
Ellipsoid	0,775-4
Random polymer coil	1,505

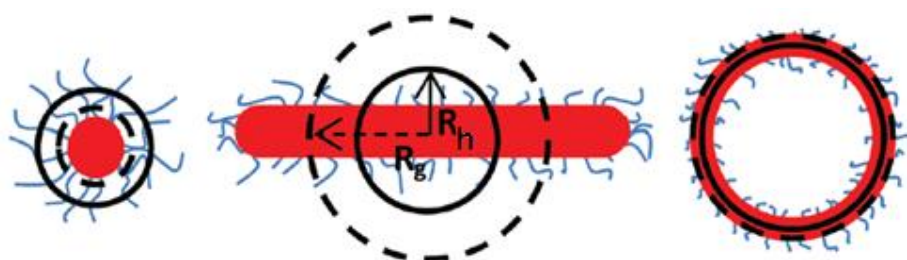


Figure 2.12 : Scheme showing how different morphology shows a different R_g/R_h , R_g is showed in dashed black line, R_h by solid black line (Patterson et al., 2014).

As shown in Figure 2.12, the difference in ρ -ratio (R_g/R_h) for spheres and polymersomes shows how the mass distributed throughout the structures and for

cylinders this ratio is related to the fact that they have reduced resistance while moving parallel to their extended axis (Patterson et al., 2014).

2.2.2.1 Static-dynamic light scattering and structural analysis

Static and dynamic light scattering methods are one of the strong tool to characterize nanoparticles, macromolecules in solution. Furthermore, this method provides information on the mass and size of the scattering particles, their polydispersity, as well as on particle-solvent interaction (Koetz and Kosmella, 2007). It is possible to get information about the conformation of the molecules in solution by using this technique.

For industrial application, native and pulp cellulose can be dissolved and processed for fiber spinning in the solvent, N-methyl morpholine N-oxide monohydrate (NMMNO). The solution state of cellulose dissolved in NMMNO was investigated by SLS. Molecular weight is obtained as several million g/mol, this high value shows that cellulose aggregates exist. The existence of aggregation is also confirmed by high value of radii of gyration. The overall scattering functions presented two partial scattering behaviour that shows bimodal distribution of the particulate state, consisting of small and large aggregates clusters (Röder and Morgenstern, 1999). Another study used light scattering proved that partial solution of native and pulp cellulose become possible in mixtures of NMMNO with diethylene triamine which permitted light scattering measurement also at room temperature (Drechsler et al., 2000).

The solution behaviour of four chitosans (91% deacetylated) with different M_w in 0,2 M CH_3COOH / 0,1 M CH_3COONa aqueous solution studied by a combination of SLS and DLS. Both SLS and DLS results show that chitosan has extended chain conformation and tend to aggregate in aqueous solution even in the presence of certain amount of low molecular weight electrolytes (Wu et al., 1995).

In other study the neutralization process of chitosan with different degrees of acetylation (DA) varies between 1% and 71% followed by SLS. Variation of R_g with pH plotted for various DAs. A sudden increase of R_g at certain pH value showed metastable state where nanoparticles formed. The plot shows that with increasing DA chitosan become more soluble in larger range of pH. Nano particles found with SLS further investigated by transmission electronic microscopy and revealed that

nanoparticles of chitosan exhibit very compact structure with approximately spherical shape (Schatz et al., 2003).

In another study, the intrinsic viscosity of amylose subfractions in three different solvent dimethyl sulfoxide, aqueous potassium chloride and potassium hydroxide investigated. These results correlated with the weight-average molecular weights and found that the polymer is in coiled conformation in each of the solvents used. Correlation of radii of gyration from light scattering with the weight-average molecular weights and plots of the angular dependence of scattering intensity also agreed with a coiled conformation (Everett and Foster, 1959).

The conformation of xanthan polysaccharide as a sodium salt in 0,1 M aqueous NaCl solution is also studied by light scattering. For this study, a series of xanthan has been prepared by ultrasonic degradation and each molecular weight fractions are collected by size exclusion chromatography and converted into the salt form. The chemical structure was confirmed by NMR, optical rotary dispersion and UV absorption spectroscopy. The radii of gyration and second virial coefficient were plotted against the molecular weight, it concluded that samples are molecularly dispersed and semiflexible macromolecules (Paradossi and Brant, 1982).

Hyaluronic acid preparation from human umbilical cord is investigated by light scattering. Firstly, hyaluronic acid in sodium form is dialyzed against to ion exchange resin to remove Na^+ ions. After that HA is investigated in aqueous solutions with different ionic strength and pH. It was found that HA behaves like a coil in neutral aqueous solution with low ionic strength. In addition molecular weight and root-mean-square-radius is determined for each different pH (Laurent and Gergely, 1955).

In another study the effect of cations on the HA from rooster comb is investigated with light scattering and viscosimetry. For this aim HA solutions in NaCl, KCl and CaCl_2 is prepared, molecular weight and R_g of HA in these solutions are obtained by light scattering. Intrinsic viscosities of HA is determined. By using the R_g , M_w and intrinsic viscosity values in the proper viscosity equation results confirmed the expanded random coil conformation of HA in these supporting electrolytes (Sheehan et al., 1983).

The coil dimension of high molecular weight bacterial sodium hyaluronate (NaHA) solution with urea in 0,2 M NaCl solutions were studied by light scattering. R_g , R_h values determined and plotted against urea concentration. The magnitude of R_g and R_h and slight decrease of R_g/R_h by the addition of urea showed that HA molecules are in extended coil conformation (Nishinari et al., 2000).

In another investigation the effect of sodium hydroxyde on the conformations, interaction, diffusion, and hydrolysis rates of HA is studied by light scattering technique. It was found that conformation, interactions and hydrolysis rates are controlled by the hydroxyl groups. Adding NaOH cause to yield $-O^-$ groups on the macromolecule. NaOH destroys hydrogen bonds and leads to quick stiffening, contraction of HA coil, a considerable decrease in intermolecular interactions and the slow intramolecular hydrolysis of glycosidic bonds (Ghosh et al., 1993).

In addition, dynamic light scattering analyses routinely used in biology laboratories to detect aggregates in macromolecular solutions, to determine the size of proteins, nucleic acids, and complexes or follow the binding of ligands. As an instance, in one study DLS is used to investigate aggregation of IgG1 monoclonal antibody at two temperatures (5°C and 25°C) under two mechanical stress conditions (shaking, stirring); another study characterized the size and stability of hepatitis A virus used to prepare vaccine (Lundblad, 2009).

2.2.3 ^1H -NMR Spectroscopy

Nuclear magnetic resonance has been considered as an advanced and information rich method for structural analysis of biomolecules, involving complex carbohydrates. With this method it is possible to determine the structural properties of molecules such as organic, inorganic, and biological molecules; furthermore obtain the molecular dynamics and conformational states (Pomin, 2013).

Characterization of the primary structure of polymers such as natural and all thermoplastic synthetic polymers is best carried out by using solution NMR methods. In addition, it is suitable for characterizing the internal dynamics of a biomolecule that is linked to its function. These dynamics are a combination of the complete molecular tumbling and the internal motion of the structural pieces and motions at various frequencies supply different types of relaxation processes (Schmidt-Krey and Cheng, 2013).

In solution NMR, biomolecules tumble freely in the more native aqueous environment, do not have to be in crystal form. However, the need of molecular tumbling in solution also cause major limitation of solution NMR. When molecules are in solid phase or highly viscous environment, in the conditions that molecules cannot tumble sufficiently, the dipole moments of excited nucleus interact strongly with each other; this leads to multiple level of resonance splitting and low-resolution spectra. These effects completely vanish if the molecule tumble fastly enough (Weingarth and Baldus, 2014).

Solution NMR methods include solutions, gels, dispersions, melts etc. Dilution, dispersion, increased temperature, etc. will provide sufficient motion into the polymer chain, in that way unwanted nuclear spin interactions can be reduced to their trace values. This will improve the ability to observe a solution NMR spectrum. (Cheng et al., 2003).

In order to understand the importance of the internal dynamics of molecules in solution NMR measurements, we should know more about the 1PULSE sequence. In NMR measurements 1PULSE sequence is used and repeated many times in order to improve signal to noise (S/N). As shown in Figure 2.13, 1PULSE sequence consists of recycle delay, pulse width and acquisition time. Pulse width is the amount of time that pulse of radiation is applied to the sample in order to excite nucleus, the excited nucleus emit radiation to turn to ground state. The decaying sin wave in Figure 2.13 shows this process of free induction of decay (FID), which is a plot of emitted radio intensity as a function of time. The time specified to acquire the FID is called acquisition time. Recycle delay is the setted time to wait for overall relaxation of nucleus and start to next pulse width (Url-3).

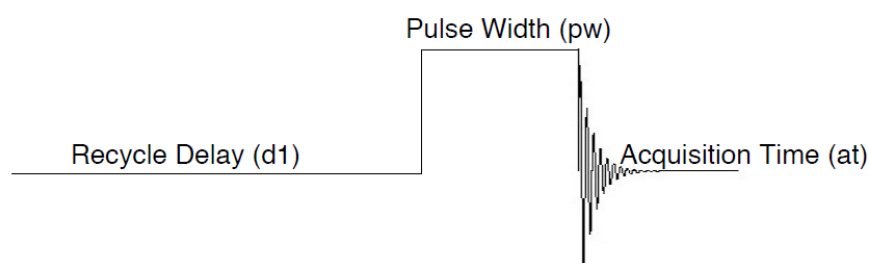


Figure 2.13 : Schematic representation of one cycle of a simple “1PULSE” pulse sequence (Url-3).

Spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2), both of them provide information about internal dynamics of solvents or polymers in gels. T_2

depends on the temperature, mobility of observed spin, presence of large molecules, paramagnetic ions and molecules. In mobile fluids, T_2 is nearly equal to T_1 , whereas in solids or slowly tumbling systems (i.e., highly viscous systems), T_2 becomes significantly shorter than T_1 , the line width of an NMR signal becomes broader. The line width of an NMR signal is determined by T_2 (short T_2 means broader lines), the maximum repetition rate during acquisition of an NMR signal is controlled by T_1 (short T_1 means signal can be acquired faster)(Url-4, Url-5).

The rate of relaxation after excitation of the nucleus essentially depends on molecular mobility. For example, methyl protons of methanol molecule are highly mobile. Therefore, spin-spin relaxation times are long, which leads to narrow NMR line widths. On the other hand, the proton mobility is low for polymers or high molecular weight solids. Low proton mobility causes the signals become broader in NMR spectra.

Polymer gels consist of mobile and immobile regions. Solution NMR gives information about the mobile region of the gels. In immobile region spin interactions are strong and lead to broad NMR line width. In order to get structural information about the immobile region, solid NMR method should be used or solution conditions (ionic strength, pH, temperature) should be changed to tune immobile regions into mobile (Osada and Khokhlov, 2001).

Figure 2.14 presents the ^1H -NMR spectrum of HA in d-DMSO. Only the peaks of anomeric protons and acetyl protons are easy to assign. The peaks at 4,54 ppm showed as N1 belongs to anomeric proton of N-acetylglucosamine (GlcNAc) unit, 4,42 ppm as U1 belongs to anomeric proton of glucuronic acid unit (GlcA). The single peak around 2 ppm belongs to the acetyl group. The remaining eight peaks on the sugar backbone are squeezed between 3,87 and 3,42 ppm and due to overlapping it is really hard to perform obtaining each peak (Pomin, 2013).

In literature, one study on HA showed that singlet peak of the methyl group of N-acetyl glucosamine in a proton spectrum presents an integrated peak area of half of the intensity expected from the known concentration of hyaluronate. The research group suggested that extreme line broadening is due to restricted segmental motion (low proton mobility) and the remainder of the signal merge into the baseline (Darke et al., 1975).

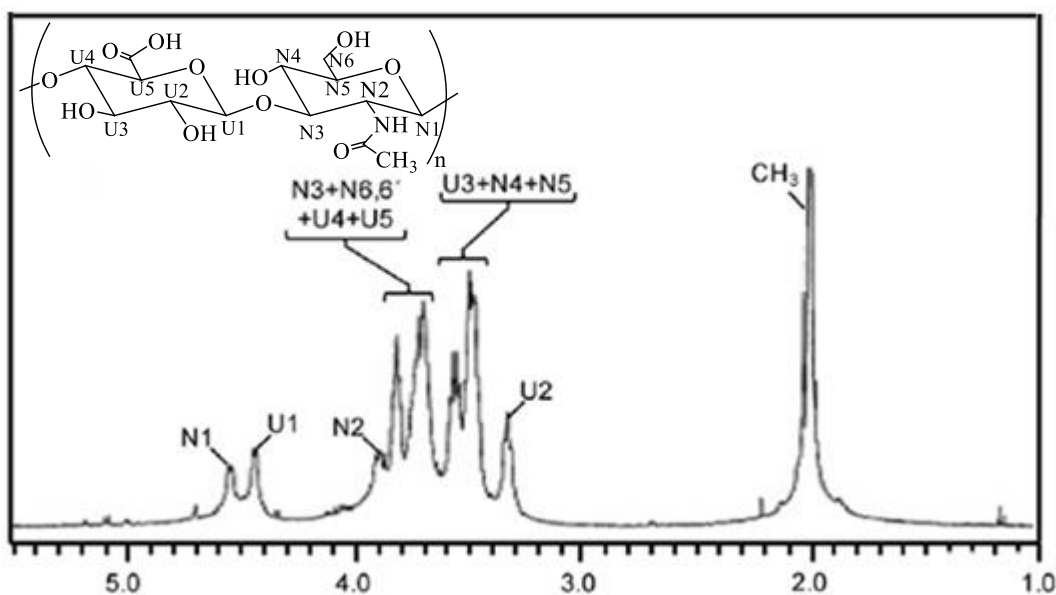


Figure 2.14 : ^1H -NMR spectrum of HA with structural scheme as an inner figure (Pomin, 2013).

Furthermore, it is possible to tune the proton mobility of HA. Previous works showed that the intensity of signals related to protons of HA depends on the concentration of NaOH and temperature for the measurements performed with ^1H -NMR (Milas and Rinaudo, 2004). With increasing temperature protons become more mobile but still interactions exist in HA solution and cannot be obtained theoretical at values between 25-85°C.

NMR method is also a beneficial tool for characterizing and quantifying chemical modifications (Schanté et al., 2011). A typical ^1H -NMR spectrum of native HA is shown in Figure 2.15a, and presents broad signals due to the highly viscous property of solution. When NaOD added to same sample, the viscosity of solution decreases and the peaks sharpen in spectrum (Figure 2.15b). In one study, L-alanine was grafted onto the carboxyl group of HA chain. Figure 2.15c shows the spectrum of final product. The new peaks at 1,33 ppm and 4,14 ppm belong to methyl group and the -CH group of L-alanine, respectively. DS% is determined by the ratios of peak integrals of the L-alanine methyl group at 1,33 ppm to the methyl protons of N-acetyl glucosamine group of HA. This NMR method for DS% calculation is useful if the modified groups are not superimposed with the peaks of native HA.

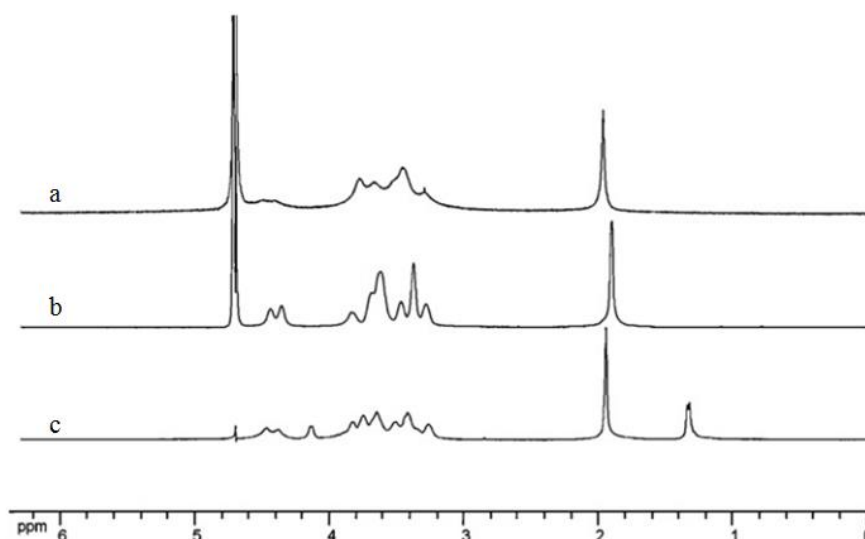


Figure 2.15 : ¹H-NMR spectra of HA in D₂O (a) and D₂O in NaOH (b) and ¹H-NMR spectrum of HA-alanine in D₂O (c) (Schanté et al., 2011).

In NMR technique, quantification can be performed by using an internal standard. In this method the area of analyte signal is compared with the standard. That is why, for accurate calculation the internal standard must have a resonance that does not overlap those of the sample. Disodium succinate can be used as an internal standard because it is soluble in D₂O, does not interact chemically with HA and gives a peak that does not interfere with other signals in the spectra.

It is known that by adding NaOD and changing temperature the line widths in spectra of HA narrow and protons become more mobile. In this study starting from this information we aim to find a relation between the proton mobility and the conformation of HA in solution.



3. EXPERIMENTAL PART

3.1 Chemicals Used

Hyaluronic acid	:Croma Pharma GmbH, Leobendorf Austria, molecular weight approximately $1,5 \times 10^6$ g/mol, analyzed with DSC as 11,9% water content
Benzylamine	: Sigma-Aldrich
DMTMM	: Sigma-Aldrich (4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride)
Sodium chloride	: Sigma-Aldrich
Sodium hydroxide	: Sigma-Aldrich
Urea	: Sigma-Aldrich
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	: Sigma-Aldrich
Sulfuric acid	: Sigma-Aldrich
Hydrochloric acid	: Sigma-Aldrich
Deuterated sodium hydroxide	: Sigma-Aldrich (NaOD 40% in D_2O)
Deuterated hydrochloric acid	: Sigma-Aldrich (DCl 20% in D_2O)
Deuterium oxide	: Euriso-top (D_2O 99.9% deuteration)
Deuterated sulfuric acid	: Sigma-Aldrich (D_2SO_4 98% in D_2O)
100mM phosphate buffer solution (PBS) with pH=6	: 3,45g of sodium $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was weighted. It was diluted with distilled water until 200 ml, NaOH added to set the pH to 6 and then added water until 250ml

3.2 Instruments

^1H -NMR	:Bruker Avance 400 MHz NMR
^1H -NMR	:Bruker Avance 250 MHz NMR
Rheometer	:Anton Paar MCR 300, cone/plate measurement, peltier temperature control
Goniometer	:ALV/CGS3 compact goniometer system with 22 mW HeNe laser, ALV-proprietary optical fiber based dedector, APD-based single photon dedector, ALV/LSE-5003 electronics and ALV-5000/ECP Correlator
Refractometer	:Carl-Zeiss refractometer with Na lamp (201W, 1,3 A, 42-60 mHZ) connected to Lauda E100 temperature control unit and Ecoline 011 batch
Magnetic stirrer	:IKA Labortechnik RCT Basic
DSC	:Netzsch STA 449 F1, Simultaneous Thermal Analysis System, Stainless steel oven, Autosampler, DSC sensor, temperature range 50°C-120°C (DSC), cooling system gaseous/liquid nitrogen

3.3 HA Solution Preparation

All weighted solid HA is corrected for the 11,9% water content. All HA solutions were studied below the critical concentration of chain overlap point. For this aim, HA solutions with low ionic strength diluted to low concentrations; HA solutions with high ionic strength diluted to higher concentrations.

Aqueous solutions with reagent at certain molarity are prepared by using sodium chloride (NaCl), sodium hydroxide (NaOH), urea ((NH_2) $_2$ CO), hydrochloric acid (HCl) and sulfuric acid (H $_2$ SO $_4$). HA solutions prepared by using these five different reagents (Table 3.1). There are some differences in the HA solution preparation methods. For this aim, solution preparation methods are explained in detail.

3.3.1 Preparation of HA solutions with NaCl solutions

6,81 mg solid HA weighted; put into vial, added 12 mL 0,075 M NaCl solution onto solid. The solution was stirred over night for achieving complete dissolution of HA. In this way 0,5 mg/mL HA in 0,075 M NaCl was prepared as a stock solution. Certain amounts of stock solution are put into different vials and 0,075 M NaCl is added onto them to obtain 0,1, 0,2, 0,3, 0,4 mg/mL HA solutions in 0,075 M NaCl. Same procedure was used for HA solutions in 0,15 M NaCl and 0,5 M NaCl, by adding 0,15 M NaCl and 0,5 M NaCl instead of 0,075 M NaCl solution (Table 3.1).

1 mg/mL HA in 1 M NaCl was prepared as a stock solution with same procedure mentioned above. Certain amounts of stock solution were put into different vials and 1 M NaCl is added onto them to obtain 0,2, 0,3, 0,4, 0,5, 0,75 mg/mL HA in 1 M NaCl.

Same procedure was used also for HA solutions in 2 M NaCl by adding 2 M NaCl instead of 1 M NaCl solution (Table 3.1).

Table 3.1 : Experimental setup for HA solutions with different ionic strength and pH.

Reagent	Reagent solution mol/L	HA conc. with reagent solution mg /ml
NaCl	0,075	0.5, 0.4, 0.3, 0.2, 0.1
NaCl	0,15	0.5, 0.4, 0.3, 0.2, 0.1
NaCl	0,5	0.5, 0.4, 0.3, 0.2, 0.1
NaCl	1	1.0, 0.75, 0.5, 0.4, 0.3, 0.2
NaCl	2	1.0, 0.75, 0.5, 0.4, 0.3, 0.2
(ND ₂) ₂ CO	0,25	0.5, 0.4, 0.3, 0.2, 0.1
(ND ₂) ₂ CO	1	0.5, 0.4, 0.3, 0.2, 0.1
(ND ₂) ₂ CO	4	0.5, 0.4, 0.3, 0.2, 0.1
DCl	0,5	0.5, 0.4, 0.3, 0.2, 0.1
D ₂ SO ₄	0,5	0.5, 0.4, 0.3, 0.2, 0.1
NaOD	0,071	0.5, 0.4, 0.3, 0.2, 0.1
NaOD	0,125	7.5, 5.0, 1.0, 0.5, 0.25
NaOD	0,25	7.5, 5.0, 1.0, 0.5, 0.25
NaOD	0,5	7.5, 5.0, 1.0, 0.5, 0.25
NaOD	1	7.5, 5.0, 1.0, 0.5, 0.25
---	---	0.5, 0.4, 0.3, 0.2, 0.1

3.3.2 Preparation of HA solutions with urea solutions

6,81 mg solid HA weighted; put into vial, added 12 mL 0,25 M urea solution onto solid. The solution was stirred over night for achieving complete dissolution of HA. In this way 0,5 mg/mL HA in 0,25 M urea was prepared as a stock solution. Certain amounts of stock solution were put into different vials and 0,25 M urea is added onto them to obtain 0,1, 0,2, 0,3, 0,4 mg/mL HA solutions in 0,25 M urea. Same procedure was used also for HA solutions in 1 M and 4 M urea by adding 1 M and 4 M urea instead of 0,25 M urea solution (Table 3.1).

3.3.3 Preparation of HA solutions with hydrochloric and sulfuric acid solutions

6,81 mg solid HA weighted; put into vial, added 12 mL ultra pure water onto solid. The solution was stirred over night for achieving complete dissolution of HA. After that certain amount of concentrated H_2SO_4 was added to solution and 0,5 mg/mL HA in 0,5 M H_2SO_4 was prepared as a stock solution. Certain amounts of stock solution were put into different vials and 0,5 M H_2SO_4 were added onto them to obtain 0,1, 0,2, 0,3, 0,4 mg/mL HA solutions in 0,5 M H_2SO_4 .

Same procedure was used for also HA solutions in 0,5 M HCl (Table 3.1).

3.3.4 Preparation of HA solutions with sodium hydroxide solutions

Stock solutions were not prepared for a set of HA in sodium hydroxide solutions. Solid HA weighted in vials, added 0,125 M NaOH onto each vial to obtain 0,25, 0,5, 1, 5, 7,5 mg/mL HA in 0,125 M NaOH. Same procedure was used for also HA solutions in 0,25, 0,5, 1 M NaOH.

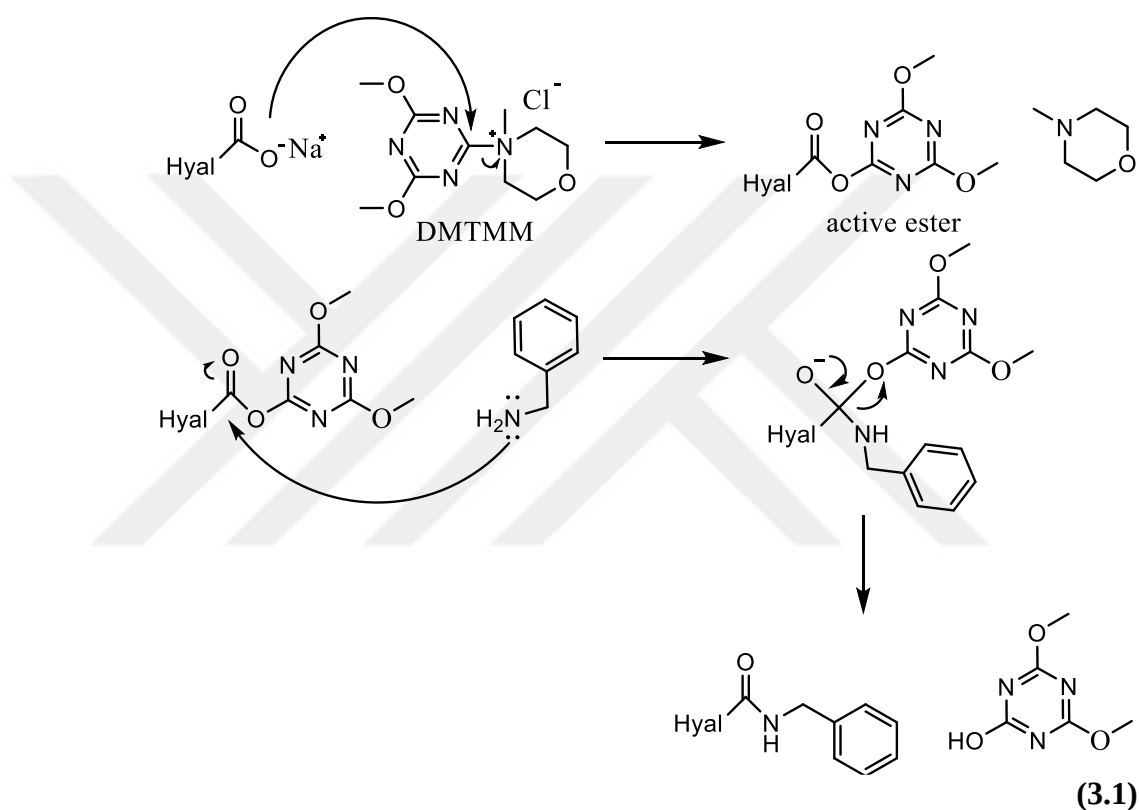
Solid HA weighted in vials, added 0,071 M NaOH onto each vial to obtain 0,1, 0,2, 0,3, 0,4, 0,5 mg/mL HA in 0,071 M NaOH.

All solutions were prepared freshly before the measurements (Table 3.1).

3.4 HA Modification with Benzylamine

Amidation of hyaluronic acid can be performed in water, at neutral pH with a water soluble carboxylic acid activator. The activation mechanism of DMTMM is show in equation 3.1.

The following general procedure was used for the preparation of hyaluronan derivate. Hyaluronan (0.59g, 1.468 mmol) was dissolved in PBS 100 mM at pH 6 until a concentration of 8,5 mg/ml (69 ml of PBS 100mM pH6). The solution was heated to 70°C. 1.35 eq of DMTMM (556.4 mg) and 2 eq of benzylamine (320 μ l) are added to the reaction. After 2 h of stirring the mixture was precipitated in 500 ml *i*-propanol. The white fibers precipitate was separated from the solution and was vacuum dried.



3.5 Light Scattering Analysis

Light scattering measurements were made with ALV/CGS3 compact goniometer system (Figure 3.1) at wavelength of 632,8 nm, and the angular distribution of scattered light was measured between 30° and 150° with respect to the direction of the beam. The sample temperature was maintained at 25°C inside a water bath.

Firstly, the aqueous solutions of different reagents filtered and the scattering measured as a baseline. After that, the aqueous solutions of HA filtered through nylon filter 0,45 μ m pore size into the cell. Measurements of diluted series of HA solutions done as 3

runs for each angle with constant laser power. The results were evaluated with ALV/ Static & Dynamic FIT and PLOT program, R_g and R_h values obtained.



Figure 3.1 : Goniometer system.

3.6 Viscosimetric Analysis

Viscosimetric measurements were carried out by Anton Paar MCR 300 Rheometer fitted in the cone/plate geometry (Figure 3.2).



Figure 3.2 : Anton Paar MCR 300 Rheometer.

All measurements were performed by 50 mm plate measuring probe with a gap between the cone and plate of 0,100 mm, the temperature was 25°C. HA is pseudoplastic it means viscosity of HA decreases with an increasing shear rate, to avoid this error shear rate was chosen as $50s^{-1}$, from the region of newtonian plateau (Figure 2.8). The viscosity of series of HA aqueous solutions (Table 3.1) measured, obtained data were plotted as η_{sp}/c against concentration c and extrapolated to $c \rightarrow 0$ for calculating intrinsic viscosity via prepared MS Excel software.

3.7 ¹H-NMR Analysis

Solution ¹H-NMR analyses were recorded on a Bruker Avance 400 MHz NMR. HA sample was dissolved in D₂O at the concentration approximately 4 mg/mL with the concentration of reagents in Table 3.1 and disodium succinate added to each solution as an internal standart.

Solid State ¹H-NMR analysis was recorded on a Bruker Avance 250 MHz NMR. HA sample was dissolved in D₂O at the concentration approximately 4 mg/mL with disodiumsuccinate as an internal standart.

3.7.1 Proton mobility calculation

Proton mobility of HA aqueous solutions with different ionic strength and pH were investigated by ¹H-NMR. Percentage of mobile protons was calculated by equation 3.2.

$$H\% = \frac{mol_{std}}{mol_s} \times \frac{A_s}{A_{std}} \times \frac{N_{prot\ std}}{N_{prot\ s}} \times 100 \quad (3.2)$$

Where mol_{std} is mol of the standart, mol_s is mol of sample, A_s is the area of the peak of interest part of the sample, A_{std} is the area of standart peak, N_{prot std} is the number of the proton of the standart, N_{prot s} is the number of proton peak of interest part of the sample.

In the equation the number of the protons were taken into account as four for standart, three for acetyl group, ten for sugar backbone and two for anomeric hydrogens.

All prepared ¹H-NMR solutions contain 0,01 mmol HA and 0,0047 mmol standart.

All proton mobility calculations were done for acetyl group, sugar bacbone and anomeric hydrogens, separately.

3.7.2 Degree of substitution calculation

DS% refers to amount of benzylamine that is copuled to HA compared to the total amount of methyl of acetyl group on HA and calculated by using equation 3.3 for modified HA samples.

$$DS\% = \frac{\text{Area of aromatic protons}}{5} \times 100 \quad (3.3)$$

The number of 5 in the equation 3.3 refers to number of benzylamine hydrogens. For all DS% calculations the acetyl peak of HA is normalized at 3 in the ^1H -NMR



4. RESULTS AND DISCUSSION

4.1 Calculation of Proton Mobility of HA Solutions

HA is a biopolymer, its aqueous solutions present highly viscous gel like behaviour. Gels consist of mobile and immobile regions. 4 mg/mL HA in D₂O was investigated with solution and solid state ¹H-NMR for confirming mobile and immobile region, respectively.

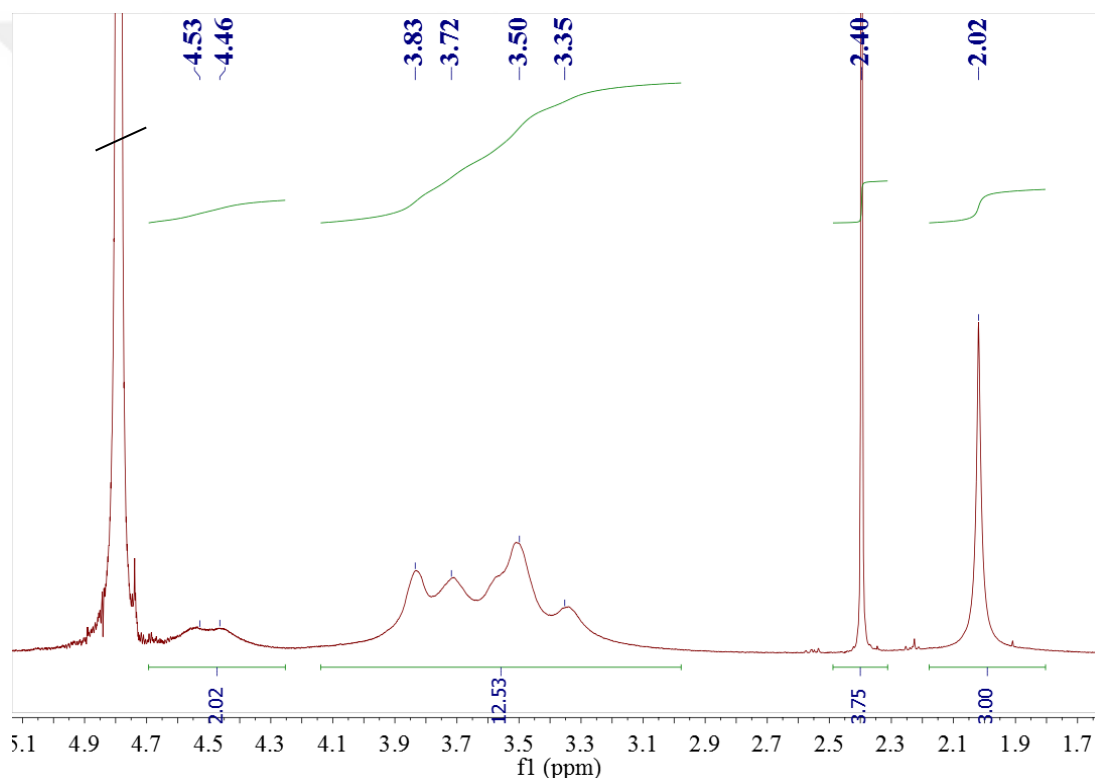


Figure 4.1 : ¹H-NMR of 4 mg/mL HA in D₂O with disodium succinate standard 25°C.

Solution ¹H-NMR measurement has been done in order to investigate mobile region of the HA solution in D₂O. Figure 4.1 shows ¹H-NMR spectrum of HA at 4 mg/mL concentration in D₂O with disodium succinate as an internal standard. The peak around 4,8 belongs to solvent and 2,4 ppm is disodium succinate peak. The signals at 4,53 and 4,46 ppm belong to anomeric protons of N-acetyl glucosamine and glucuronic acid respectively. The signal at 2,02 ppm belongs to methyl group of

N-acetyl glucosamine. Remaining ten peaks of hydrogens on the sugar backbone are squeezed between 3,0 and 4,1 ppm.

As shown in Figure 4.1, HA spectrum consists of three different group of peaks belong to acetyl group, sugar backbone and anomeric hydrogens. In spectrum, signals are broad and the reason is more likely the shorter spin-spin relaxation time of HA caused by non-bonded interactions, which lead to high viscosity and stiffen the chain. In this spectrum all protons are not visible, the immobile region protons of HA solution cannot be observed.

Solid-state ^1H -NMR measurement of 4 mg/mL HA in D_2O with disodium succinate standard was taken in order to confirm the immobile protons of HA solution that is not visible by solution ^1H -NMR. As seen in Figure 4.2, it is possible to observe peaks are generated by HA. The peaks between 2 and 4,6 ppm belongs to acetyl, sugar backbone and anomeric hydrogens of HA; between 4,8 and 5,6 ppm to hydroxyl hydrogens of HA and water. The immobile region of HA solution in D_2O is confirmed with this analysis.

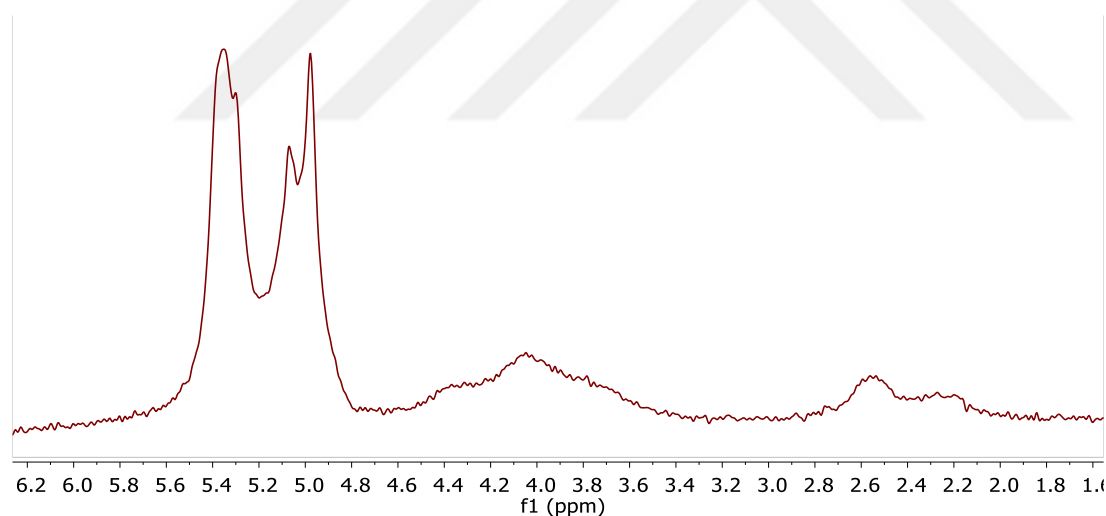


Figure 4.2 : Solid state ^1H -NMR of 4 mg/mL HA in D_2O with disodium succinate standard 25°C.

In order to covert this immobile region's protons to mobile, different reagents are added to HA solutions and their effects on the proton mobility are investigated. We studied with NaOH, NaCl, urea, H_2SO_4 and HCl for this aim.

For the ^1H -NMR spectra are shown in Figure 4.3, Figure 4.4, Figure 4.5; two stacked spectra the signal intensity was normalized with the signal of the standard disodium succinate.

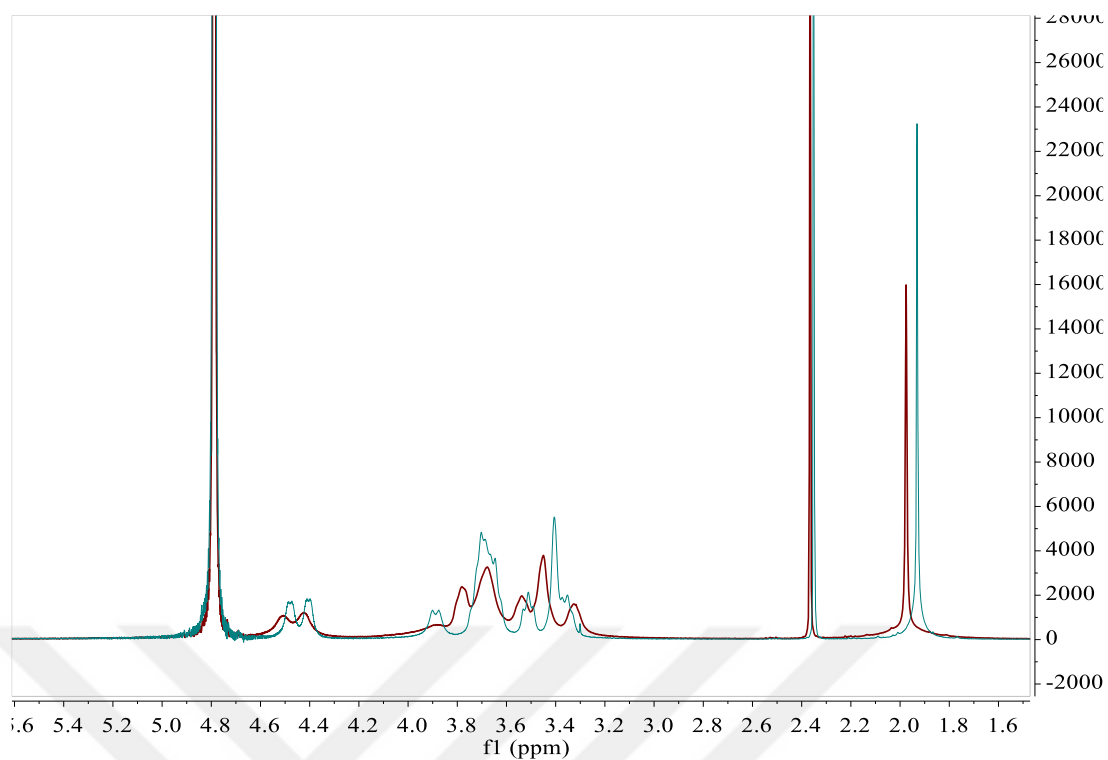


Figure 4.3 : ^1H -NMR spectra of HA in 0,071 M NaOH (red) and 0,25 M NaOH (blue).

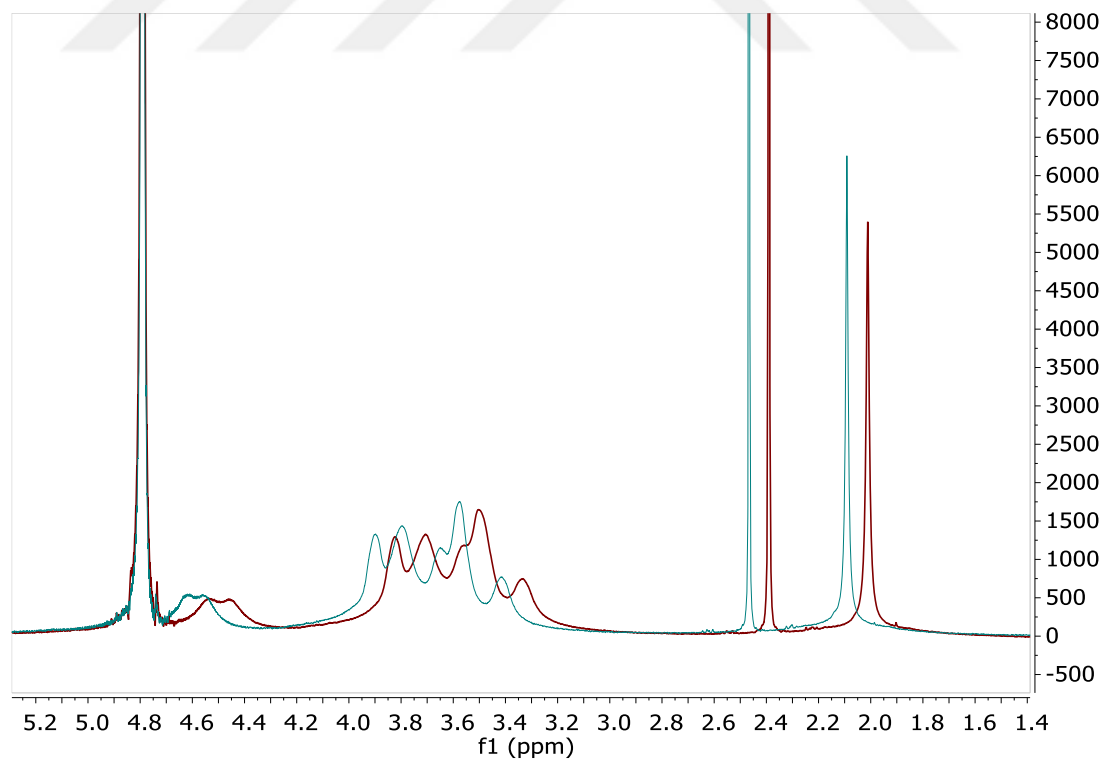


Figure 4.4 : ^1H -NMR spectra of HA in 0,15 M NaCl (red) and 0,5 M NaCl (blue).

Figure 4.3 shows NMR spectra of HA in 0,071 M NaOH and 0,25 M NaOH. When the alkali concentration increases, the spectra shifts to up fields; the peak of anomeric protons and sugar backbone protons become sharper. As it can be seen from this figure the area under the peaks of anomeric, acetyl and sugar backbone protons increased by alkali condition.

Spectra of HA in two different concentration of urea solutions are shown in Figure 4.5. It is clear that higher urea concentration causes the intensity of peaks to increase. However, when these two stacked spectra compared to with alkali and higher ionic strength conditions, urea causes really low increment of peak areas and broad peaks are present.

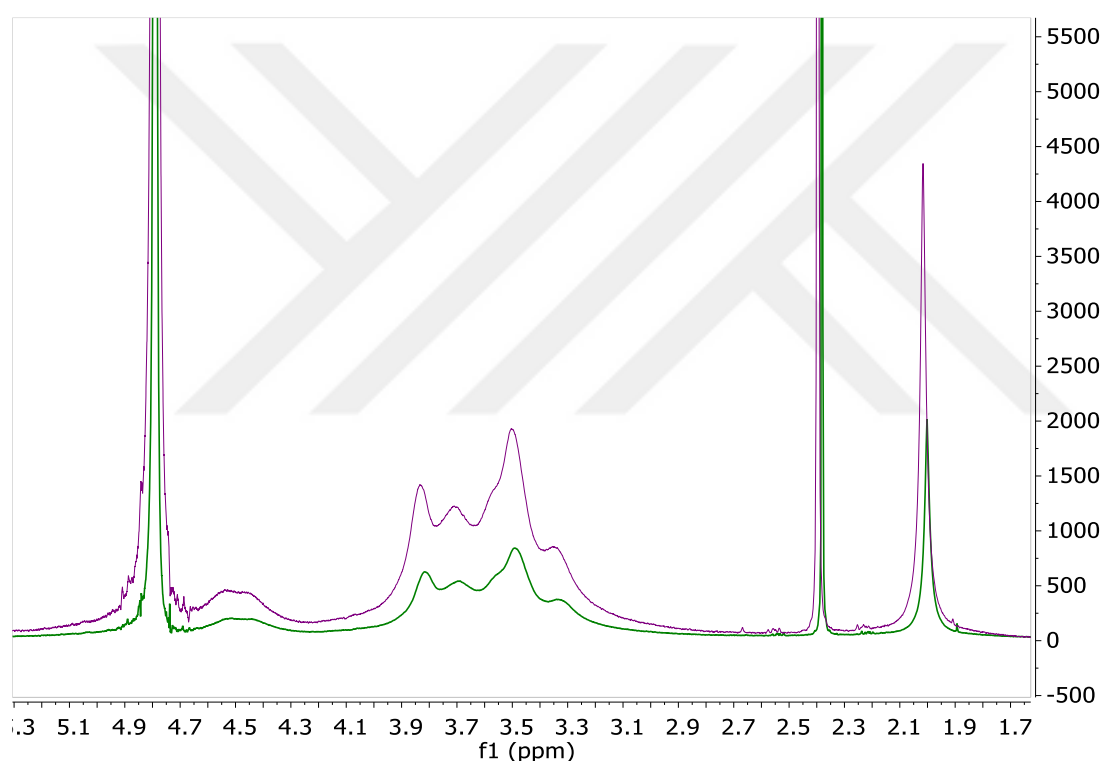


Figure 4.5 : ^1H -NMR spectra of HA in 0,25 M urea (green) and 1 M urea (purple).

Proton mobility is calculated from the obtained spectra. For an example, proton mobility of acetyl group of HA in 0,5 M NaOH (Figure 4.6) was calculated as described. The areas of peaks are integrated in program and found 3 and 1,91 for the acetyl and standard protons, respectively. For the proton mobility calculation equation 4.1 was used. The values of the peak of acetyl and standard, mol of HA and standard put into the equation and the proton mobility calculated for acetyl group.as :

$$H_{ace} \% = \frac{mol_{std}}{mol_{ace}} \times \frac{A_{ace}}{A_{std}} \times \frac{N_{prot std}}{N_{prot ace}} \times 100 = \frac{0,0047}{0,01} \times \frac{3}{1,91} \times \frac{4}{3} \times 100 = 98,9 \quad (4.1)$$

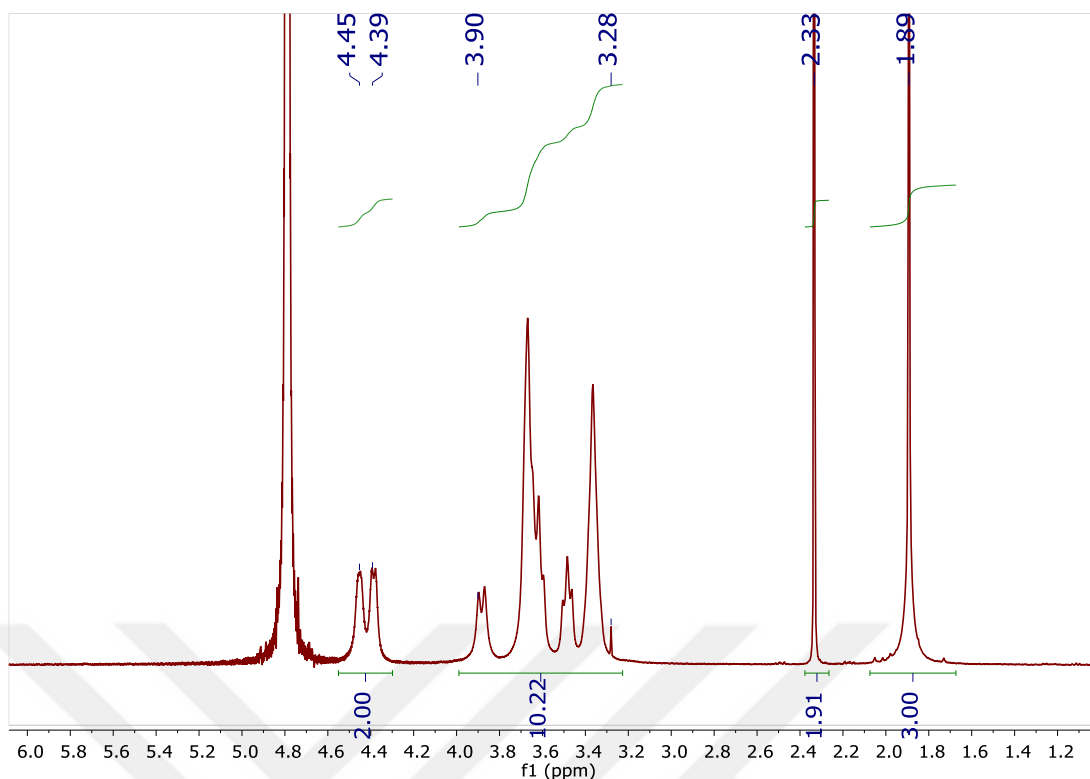


Figure 4.6 : ^1H -NMR spectrum of HA in D_2O with 0,5 M NaOH.

Proton mobility of HA changes with the concentration and type of the reagents (Table 4.1). In order to compare the reagents effect on the proton mobility, the acetyl group's proton mobilities in Table 4.1 are plotted against to reagent concentration in Figure 4.7.

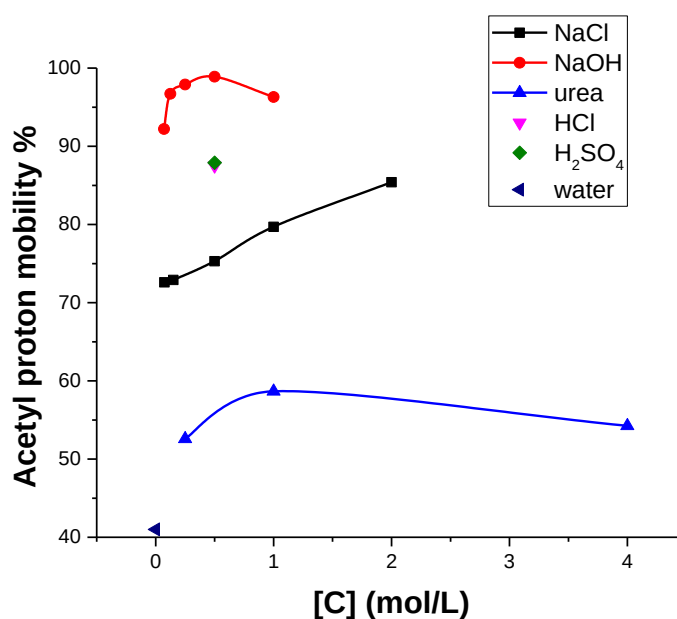


Figure 4.7 : Percentage of proton mobility of acetyl groups as a function of NaCl, NaOH, urea, H_2SO_4 and HCl concentration.

Table 4.1 : Proton mobility of 4 mg/mL HA in D₂O with various reagents.

Reagent	Concentration mol/L	Proton mobility of acetyl group	Proton mobility of sugar backbone	Proton mobility of anomeric protons
NaOD	0,071	92,2	94,0	83,3
NaOD	0,125	96,7	97,2	87,7
NaOD	0,25	97,9	98,3	93,1
NaOD	0,5	98,9	97,7	98,5
NaOD	1	96,3	95,8	92,1
NaCl	0,075	72,6	75,1	59,1
NaCl	0,15	72,9	76,8	59,3
NaCl	0,5	75,3	80,7	60,4
NaCl	1	79,7	82,8	58,9
NaCl	2	85,4	82,9	-
Urea-d ₄	0,25	52,6	66,2	49,7
Urea-d ₄	1	58,7	68,8	51,1
Urea-d ₄	4	54,3	66,1	46,5
DCl	0,5	87,5	93,2	82,7
D ₂ SO ₄	0,5	87,9	92,2	81,3
---	---	52,9	61,6	41,7

Figure 4.7 shows that acetyl proton mobility reaches highest values with addition of NaOH to the samples. Sulfuric acid, hydrochloric acid, sodium chloride and urea follow the row respectively.

When the concentration of NaOH increases acetyl protons become more mobile .The reason is that by adding NaOH to HA solution in D₂O, hydrogen bonds are broken down. Without non-bonding interactions protons tumble more quickly and this leads to narrow spectral lines, whereas slow motions may cause very broad lines for detecting with conventional high resolution spectroscopy (Darke et al., 1975; WELTI et al., 1979). The highest acetyl proton mobility were reached in 0,5 M NaOH. However, in 1 M NaOH proton mobility decreases, this effect may be caused by decomposition of HA under strong alkali condition. Strong alkali conditions produce a decrease in the area of the acetyl peak of HA and also in proton mobility.

Adding NaCl to HA solution increases the proton mobility. Na⁺ ions screen the electrostatic repulsion between the negatively charged groups on HA chains. On the

other hand, increasing salt concentration saturates the water and this condition may lead the HA chains to form intramolecular hydrogen bonds instead of hydrogen bond with water molecule. All these conditions decrease the dimension of HA chains and increase the acetyl proton mobility.

Urea also increases the acetyl proton mobility but not as much as other reagents. Urea is known as a hydrogen bond breaking agent, disrupts the intermolecular hydrogen bonds of HA by forming new hydrogen bond on itself. This effect is not strong enough to make protons mobile more than 59%.

In addition, proton mobility of sugar backbone and anomeric hydrogens are also investigated as a function of reagent type and concentrations (Figure A.1 and Figure A.2). Consequently, for each proton groups similar behaviour with small deviations are determined. However, due to the fact that investigating proton mobility of each group would be complicated, this study focused on acetyl group since for the DS% calculations of hyaluronic acid, acetyl groups are used as an internal standard (Lee et al., 2008; Luo and Prestwich, 1999; Pouyani and Prestwich, 1994). Hereafter, acetyl hydrogen's mobility is mentioned as "proton mobility". Sugar backbone and anomeric proton mobility is stated particularly.

4.2 SLS-DLS Analyses

4.2.1 Effects of reagents on radius of gyration

R_g is used to describe the dimension of the polymer chain in solution and measured by using static light scattering technique. R_g corresponds to the mean distance of one scattering center within the particle, from the center of the particle as a whole. This parameter contains information about size of the macromolecule, furthermore about the conformation of polymers in solution by comparing with the R_h values (Patterson et al., 2014).

R_g values are obtained by static light scattering measurements. Data is evaluated by Berry plot via extrapolating obtained data to zero angle and zero concentration (Figure 4.8). The Zimm plot is used for mid-size particles 20-50 nm, whereas Berry plot is useful for large polymers with size bigger than 50 nm (Url-7). All the calculations of R_g were made by the berry plot. Berry plot of high molecular weight HA (HA studied 1.5×10^6 g/mol) gives better result with first order concentration fit.

Besides, second degree of angular fit used due to existence of curvature in angular plot for each concentration. (Dupont and others, 2003; Potts, 1993).

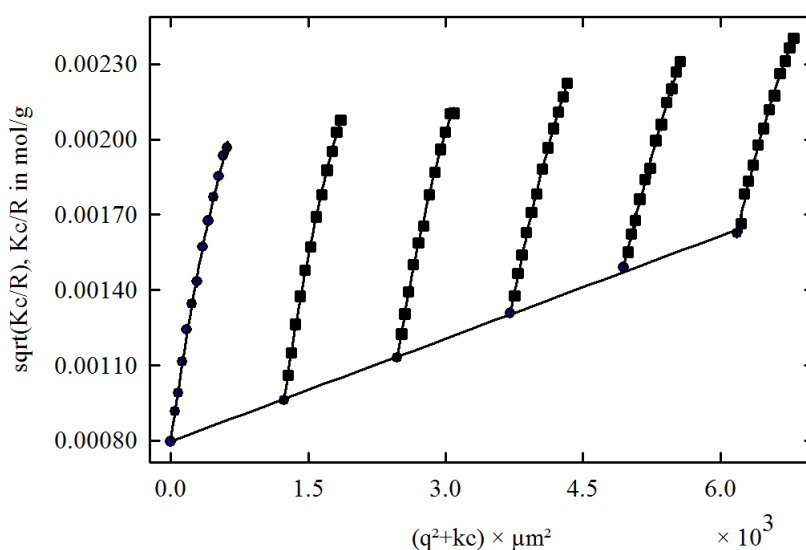


Figure 4.8 : Typical Berry plot of light scattering data at 25°C from hyaluronic acid solutions in 0,5 M NaCl. ■, observed data; ● extrapolated.

Obtained R_g values are plotted against reagent concentration of HA solutions. A relation between reagent concentrations and the R_g can be observed from the Figure 4.9. Figure 4.9 shows that each reagent has different effects on R_g .

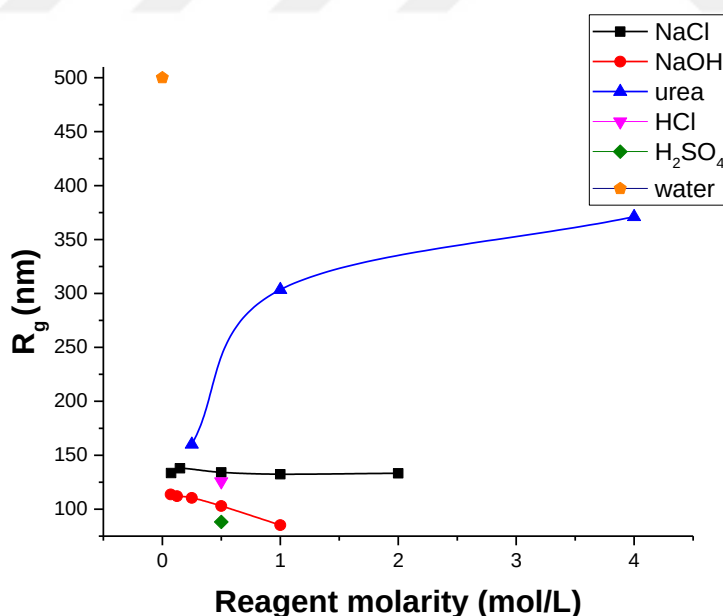


Figure 4.9 : Relation between reagent molarity and radius gyration.

R_g decreases due to both alkali effect and ionic strength, but mainly with different mechanism (Cowman and Matsuoka, 2005; Ghosh et al., 1993). Alkali effect causes breakage of hydrogen bonds that stabilize the structure of HA in solution, secondary

structure of HA collapsed, chains contract and R_g decreases. Furthermore, alkali effect causes slow intramolecular cleavage of glycosidic bonds. Unlike alkali effect, increasing salt concentration (ionic strength) does not cause glycosidic bond cleavage, just screen the electrostatic repulsion between HA residues (negatively charged groups on the structure), effects the interchain stability caused by H-bonds and reduce the size of the chains, consequently R_g value (Fouissac et al., 1992; Napier and Hadler, 1978; Sheehan et al., 1983).

In this study, HA samples with NaOH were prepared freshly before the measurements in order to avoid hydrolyses effect of NaOH. In this condition, it was easy to ignore the decrease in M_w of HA caused by alkali effect. Figure 4.9 presents that high pH has much greater effect than electrostatic shielding on the contraction of the size of HA. The reason is that alkali condition provides higher shielding of ionic charge on the polymer chain than salt and cause rapid breakage of hydrogen bonds; as a result more contracted structure obtained with alkali.

Furthermore, lowering pH cause also decrease in R_g . Sulfuric acid causes stronger contraction (88,17 nm) compare to hydrochloric acid (125,6 nm). It is known that HA is degraded in acid solutions (Florkin et al., 1971; Tokita and Okamoto, 1995; Tømmeraas and Melander, 2008). Furthermore, when comparing the degradation study of HA in same molar H_2SO_4 and HCl at same temperature, the disaccharide formation is more with H_2SO_4 while higher molecular weight fractions more with HCl (Inoue and Nagasawa, 1985). In this study, experiments with two different acid were conducted at room temperature and same length of time (half day), it is convenient more contracted structure was obtained with sulfuric acid.

Solutions with urea show different behaviour than other reagents. By adding small amount of urea (0,25M), R_g decreases sharply compare to HA solution in only water. It may caused by bivalent binding of urea to HA chains and cross-link the chains which decreases the R_g . However, more concentrated urea forms aggregates and they led to larger R_g . Results are in a good agreement with literature (Sagle et al., 2009).

4.2.2 Effect of reagents on hydrodynamic radius

R_h is used to describe the dimension of polymers in solution and calculated by dynamic light scattering (DLS). R_h is a value that refers to how particle diffuses within a fluid so it is referred to as hydrodynamic parameter. The radius obtained by

this technique is the radius of sphere which has same brownian motion as the polymer chain in solution studied (Patterson et al., 2014).

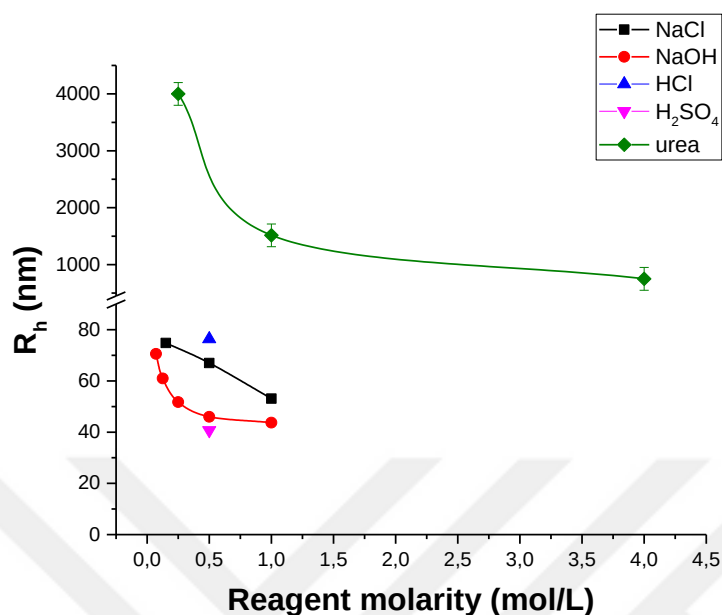


Figure 4.10 : Relation between reagent molarity and hydrodynamic radius.

Figure 4.10 shows the relation between hydrodynamic radius and different concentration of reagents (NaCl, NaOH, urea, H₂SO₄ and HCl). The R_h values of HA in water is 4800 nm, in acidic conditions R_h decreases. H₂SO₄ causes more decrease of R_h than HCl because of the same reason mentioned in previous chapter. Increasing alkali concentration causes contraction of the structure of HA and decreases the R_h sharply. Increasing salt concentration decreases R_h , but not as strong as the alkali condition. Some R_h values in salt solution were ignored in the graph for some concentrations because of high deviation from the general trend.

It is expected with increasing salt and alkali concentration R_h values decrease, since in these conditions HA chains contract, dimension of polymer decreases. The contracted structure of HA loses its highly viscous, gel like behaviour. HA polymer chains cannot interact each other strongly, like forming hydrogen bonds which stabilizes the structure of HA and form meshwork; and act like independent small molecules. As a result smaller R_h values were obtained for contracted structure of HA in solution.

R_h values of HA are 4000, 1515 and 750 nm in 0,25 M, 1 M and 4 M urea solutions, respectively. The R_h of HA decreases with the increase of urea concentration, that

can be caused by the break of intermolecular hydrogen bonds by urea. It is obvious urea causes less contraction than other reagents. Whereas R_g of HA increases with increasing urea concentration, R_h decreases. Urea disrupt intermolecular hydrogen bonds, but not effectively the intramolecular one. Stronger intramolecular hydrogen bonds may cause HA chains to gather center, like sphere conformation, but on the other hand breaking of intermolecular bond may provide the molecules a bit more freely motion in the solution, as a result lower hydrodynamic volume. Consequently, different behavior of R_g and R_h may show the conformation change of HA in solution.

4.2.3 Relation between R_g , R_h and proton mobility

Obtained R_g values are plotted against to proton mobility of HA solutions (Figure 4.11). A relation between proton mobility and the R_g can be observed from the graph.

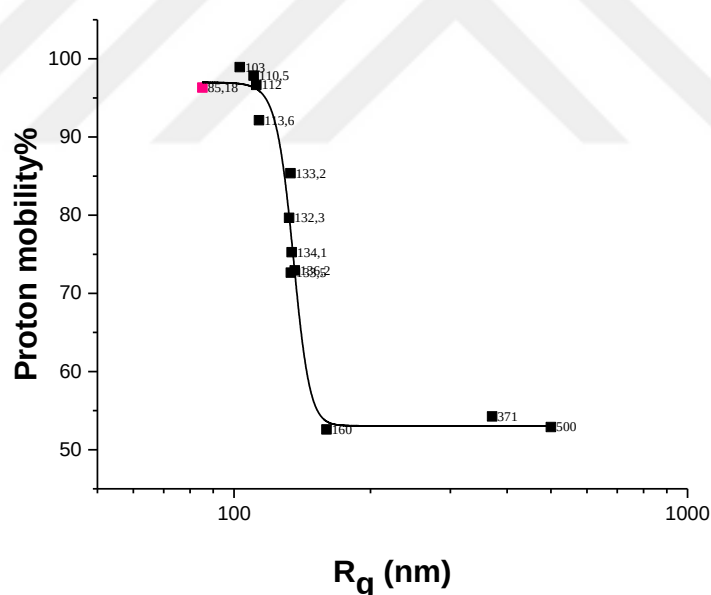


Figure 4.11 : Relation between radius of gyration and proton mobility %.

Figure 4.11 shows that while R_g of HA solutions decreases, the proton mobility increases. As polymer's dimension becoming smaller in solution, hyaluronic acid solution loses its highly viscous character by change of conformation in a way that hydrogens on the polymer become more mobile. The highest mobility is reported at the 103 nm R_g . When the R_g values are smaller than 103 nm, proton mobility decreases because of degradation of HA caused by strong alkali condition. Between

112 and 160 nm radius, proton mobility decreases sharply; this behaviour points out the transition from more stiffened and suppressed structure to the extended form. HA with extended structure forms inter- and intramolecular hydrogen bonds, chains interact each other and forms a network (gel). This highly viscous solution of hyaluronic acid shows lower proton mobility, because the hydrogen atoms cannot tumble freely in the solution and highly viscous medium causes spin interactions, as a result broadened spectra.

The structural transition as a function of R_g is presented in Figure 4.12, by using the data from the Figure 4.11. As can be seen, with R_g values between 103-112 nm HA chains have suppressed, contracted coil structure. Chains do not effectively interact with each other and move in the solution more freely. Between 112-160 nm, HA chains have more extended form but do not interpenetrate completely, the network has similar structure to island-type, where some areas are separated by significant distance. However while R_g increases from 112 nm to 160 nm, chains extend in solution, start to touch and overlap. Over 160 nm chains interpenetrate and intermolecular interactions are present.

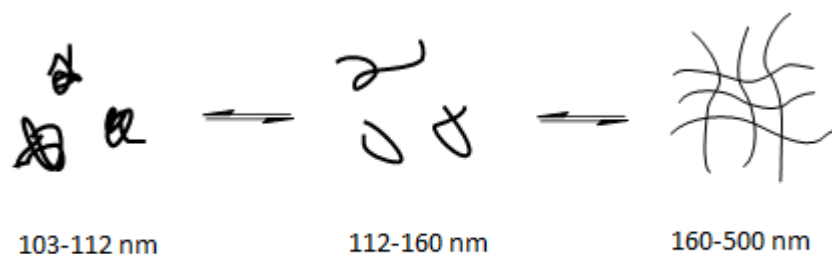


Figure 4.12 : Schematic presentation of conformation of HA as a function of R_g .

Obtained R_g values are also plotted against proton mobility of sugar backbone and anomeric hydrogens (Figure B.1 and Figure B.2). Similar relation between proton mobility of acetyl hydrogens and R_g is found with small deviations for anomeric and sugar backbone hydrogens.

As it is shown in Figure 4.13, there is inverse correlation between proton mobility and hydrodynamic radius of HA solutions. The maximum proton mobility is observed in the graph at 46 nm R_h . The results show that there is a small change in R_h of HA with decreasing proton mobility, whereas R_g showed greater change as a function of proton mobility.

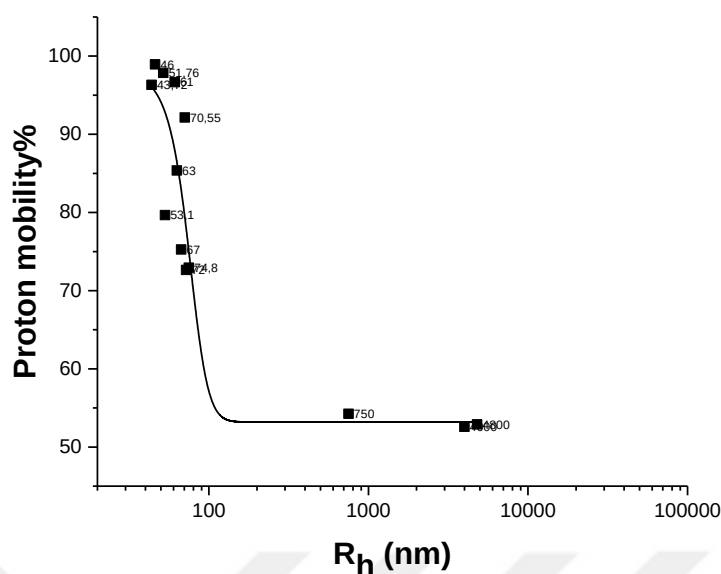


Figure 4.13 : Relation between hydrodynamic radius and proton mobility %.

When we compare the Figure 4.11 and Figure 4.13, similar behavior of R_g and R_h with changing proton mobility is obtained.

R_h values are plotted against proton mobility of sugar backbone and anomeric hydrogens (Figure B.3 and Figure B.4). Similar relation as between proton mobility of acetyl hydrogens and R_h was found with small deviations for anomeric and sugar backbone hydrogens. However, deviation of anomeric hydrogens were higher than sugar backbone hydrogens.

In this study it was observed direct correlation between proton mobility and polymer dimension in solution. These results provide proton mobility information that is independent of added reagent type. With this information we can predict the proton mobility of same or similar polymers in different solvents, just by measuring the polymer dimension in that solvent.

4.2.4 Relation Between Conformation and Proton Mobility

In order to obtain conformation of HA in solution, the rate of R_g/R_h is calculated. R_g/R_h is plotted versus proton mobility (Figure 4.14).

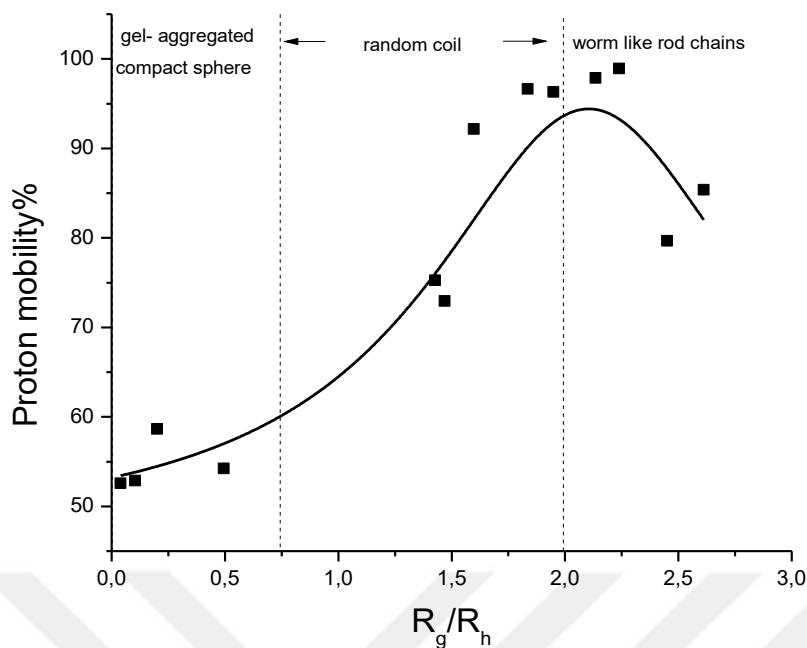


Figure 4.14 : Relation between R_g/R_h and proton mobility %.

The R_g/R_h value between 0-0,5 shows the aggregated compact sphere or gel like structure. This behaviour causes HA chains in the solution to have lower proton mobility because of the inter- and intra-molecular hydrogen bonds that restrict the molecular motion. In the random coil conformation as the R_g/R_h ratio increases proton mobility increases. The maximum proton mobility observed with worm like rod chains conformation at 2,24 R_g/R_h ratio. While R_g/R_h value increases above the 2,24, the conformation of HA turns into rigid rod, the decrease in the proton mobility was observed. This may be caused by the aggregation of HA chains. In literature it is known that polyelectrolytes with rigid-rod conformation have a tendency to form aggregates (Litt et al., 2004; Liu et al., 1998). As a result, HA in solution shows the maximum mobility in worm like rod chains conformation.

4.3 Viscometric Analyses

4.3.1 Effect of reagents on intrinsic viscosity

The intrinsic viscosity is a measure of the hydrodynamic volume of HA chain in the solution. By using intrinsic viscosity, it is possible to estimate molecular parameters such as the time-average and ensemble average hydrodynamic volume of HA, or

persistence length of the chain, both are measures of the apparent stiffness of the chain and it is consequent expansion (Cowman and Matsuoka, 2005).

Results show that relation between reagent concentration and intrinsic viscosity is correlated to those that R_g and R_h (Figure 4.15). This result is anticipated, since intrinsic viscosity is related to hydrodynamic radius, it changes parallelly (Url-8).

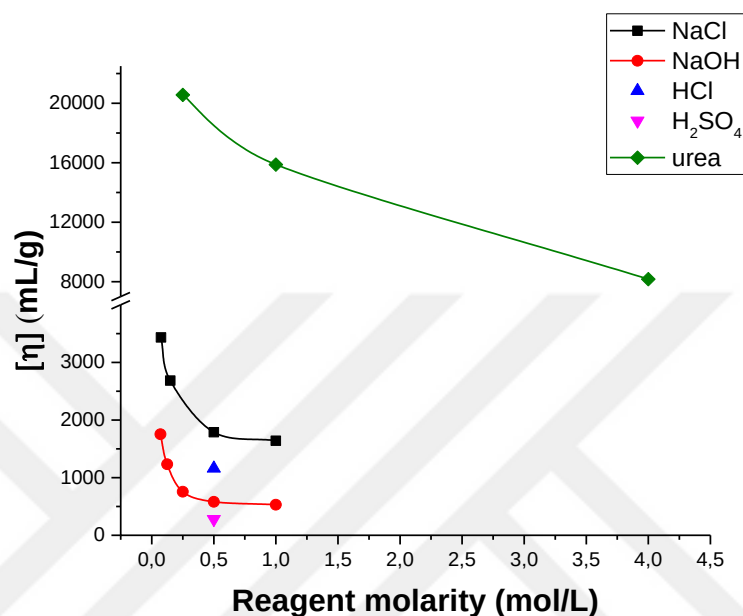


Figure 4.15 : Relation between reagent concentration and intrinsic viscosity

Increasing pH breaks inter- and intra-molecular hydrogen bonds, induce the contraction of chains and decrease the R_g and R_h . As a result, decreased dimension of the polymer coils occupy small volume in the solution and intrinsic viscosity decreases, HA solutions become more fluid. In Figure 4.15, it is obvious that NaOH leads to highest contraction on the structure of HA among NaCl, HCl and urea.

Viscosimetry data in acidic form are convenient with R_g and R_h values, H_2SO_4 leads intrinsic viscosity to decrease more than HCl. Increasing ionic strength also decreases the intrinsic viscosity. Increasing ionic strength suppress the electrostatic repulsion, R_g and R_h decreases, it means the dimension of HA chain in solution decreases, as a result intrinsic viscosity decreases. However, as seen from Figure 4.15 screening the electrostatic repulsion does not affect the intrinsic viscosity as strong as alkali and acidic conditions.

HA in urea solution shows highest intrinsic viscosity among other reagents (Figure 4.15). This result is expected because highest R_h and R_g values of HA were

obtained in urea solutions. As seen from Figure 4.15, even though urea disrupt the intermolecular hydrogen bonds, does not affect the viscosimetric behavior of HA solutions as strong as other reagents studied. Compatibly with R_h values, intrinsic viscosity shows same behaviour with changing proton mobility. Decreasing hydrodynamic radius result as a decreasing intrinsic viscosity.

4.3.2 Relation between intrinsic viscosity and proton mobility

Intrinsic viscosity gives us information about effective specific volume (in units of mL/g) of the domain of a macromolecule in solution.

It is possible to obtain critical concentration from intrinsic viscosity with the equation 2.13. Critical concentration is the concentration which polymer fills the volume of solvent totally. Below the critical concentration, polymer coils do not interact strongly, because the polymer coil per unit of solvent is very low, coils move freely. At critical concentration, polymer coils fill the volume of solvent totally and start to touch and overlap. Over the critical concentration, coils interact each other and form a network (Figure 4.16).

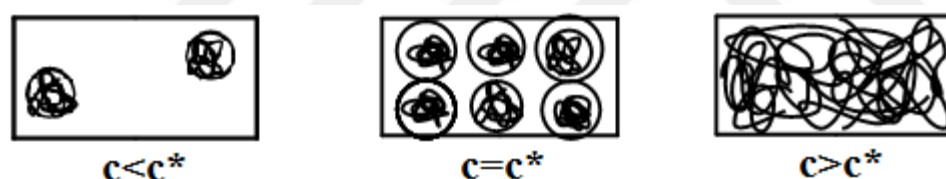


Figure 4.16 : Schematic presentation of the regions above, below and equal to critical concentration.

Intrinsic viscosities plotted against proton mobility (Figure 4.17). Results showed that the relation between proton mobility and intrinsic viscosity is similar to those that R_g and R_h .

In Figure 4.17, we can easily observe that above the intrinsic viscosity 1233,3 mL/g the proton mobility of HA starts to decrease sharply. This turning point shows us one change in the solution properties. After 1233,3 mL/g HA coils get extended form in the solution and start to touch and overlap (condition as critical concentration), this effect causes proton mobility to decrease. It may be said that 1233,3 mL/g is chain overlap point of HA, above this value HA has extended form and chains interact. As HA chains extend the intrinsic viscosity increases and related to this condition proton mobility decreases.

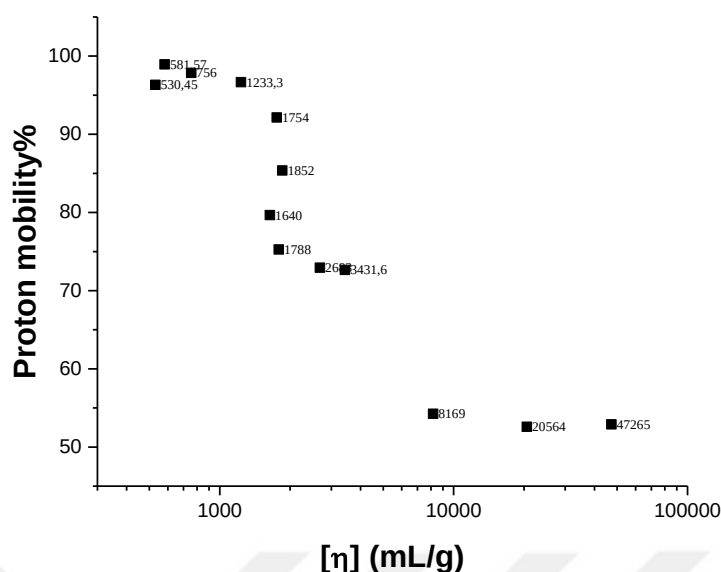


Figure 4.17 : Relation between intrinsic viscosity and proton mobility %.

When we use intrinsic viscosities in equation 2.13, it is obvious that the HA in solution with lower intrinsic viscosity has higher critical concentration. This result shows that for the HA in solution with lower intrinsic viscosity, the sample concentration studied by ^1H -NMR was below the chain overlap point and polymer chains can move freely, as a result HA showed high proton mobility. On the other hand, the HA in solution with higher intrinsic viscosity has lower critical concentration, that shows the concentration studied by ^1H -NMR was above the chain overlap point and HA coils interact with each other, form network. In this condition, HA chains form intermolecular hydrogen bonds, protons cannot tumble freely and proton mobility decreases.

In addition, proton mobility of sugar backbone and anomeric hydrogens were plotted against intrinsic viscosity, same relation between proton mobility of acetyl hydrogens and intrinsic viscosity was found with small deviations for sugar backbone and anomeric protons (Figure C.1 and Figure C.2).

4.4 Effect of Proton Mobility on Degree of Substitution Calculation

The substitution reaction of HA with benzylamine was chosen in order to investigate the effect of proton mobility on the DS% calculation. In the ^1H -NMR spectrum of benzylamine modified HA in D_2O (Figure 4.18) new signals formed which belong to

the benzylamine. These signals are the two signals squeezed between 7,2 and 7,6 ppm belong to aromatic protons and the signal around 4,2 ppm belongs to methylene group of benzylamine. Furthermore, the anomeric, sugar backbone and acetyl group hydrogens of HA are observed in the spectrum.

The DS% of benzylamine modified HA in D₂O is found 23% by using equation 3.3. However, due to the broad signals in the spectrum it would not be possible to determine accurately DS% by using acetyl peak as a reference, because it is obvious that HA protons are not fully mobile and will cause some error in the DS% calculations.

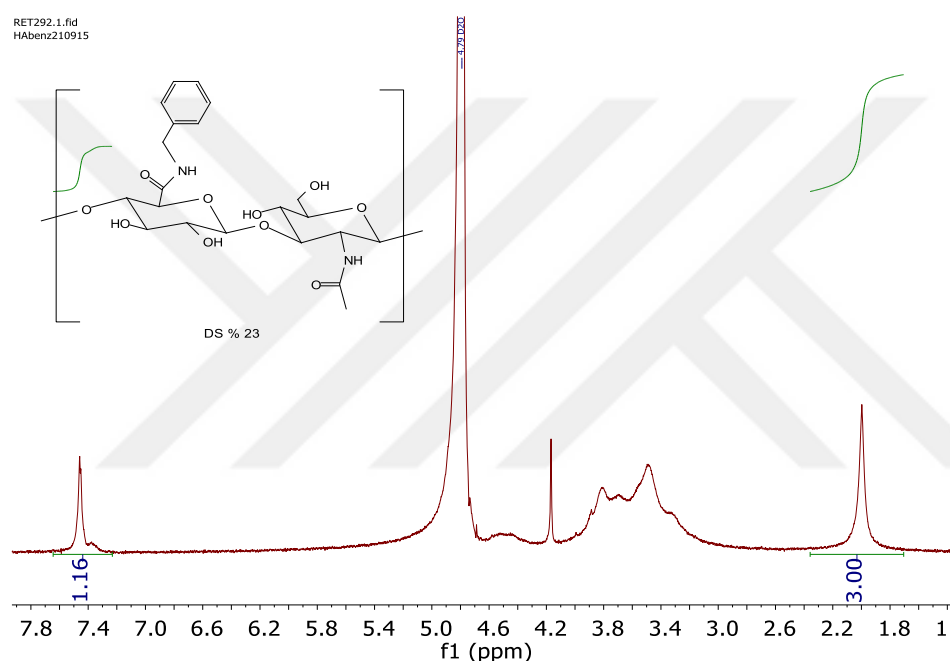


Figure 4.18 : ¹H-NMR spectrum of benzylamine modified HA in D₂O.

For accurate DS% calculation we choose D₂O with 0,5 M NaOD as a solvent because the maximum proton mobility of HA is observed in that condition. Thus, same sample dissolved in D₂O with 0,5 M NaOD and DS% was calculated as 15%. These two different results were caused by different proton mobility of HA. The DS% result as 15% is correct because in the 0,5 M NaOD protons of HA are fully mobile. The protons of HA used as reference in the ¹H-NMR has same mobility as the protons of the grafted molecule. In the spectrum there are no broad peaks caused by strong spin interactions, all peaks are sharp and it is easy to integrate peak areas accurately for calculations (Figure 4.19).

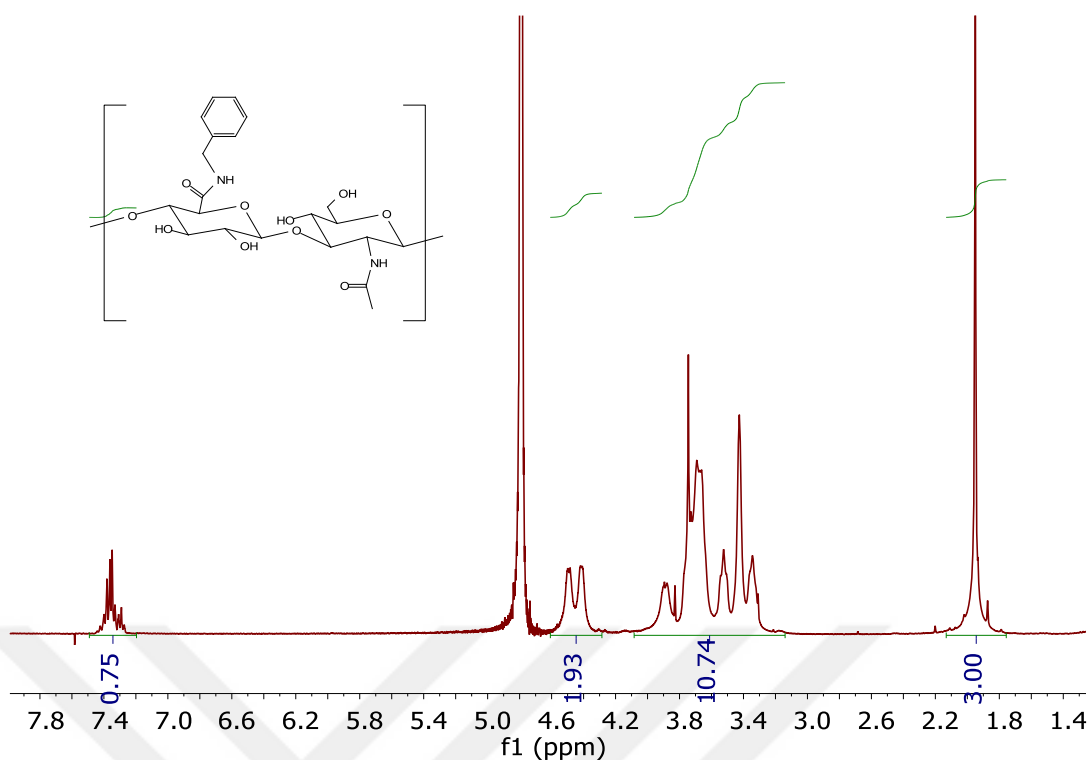


Figure 4.19 : ^1H -NMR spectrum of benzylamine modified HA in D_2O with 0,5 M NaOD.

Proton mobility is very important because it affects the result of DS% calculation, therefore different mobility of the protons, different degree of substitution was obtained by ^1H -NMR method. Due to easy sample preparation, high reproducibility, quick measurement and accurate results with full proton mobility; this ^1H -NMR method would be practical for DS% calculations.



5. CONCLUSION

Proton mobility of HA solutions with different reagents such as NaOH, NaCl, urea, H₂SO₄, HCl were investigated. The maximum proton mobility was observed in the HA solution with 0,5 M NaOH.

R_g and R_h values were obtained with SLS and DLS, plotted against to proton mobility. Results showed the dimension of the HA polymer chains in solution have a correlation with the proton mobility. Decreasing the dimension of the polymer in solution, proton mobility increased. It is obvious that by decreasing radius polymer chains cannot interact effectively, inter- and intramolecular hydrogen bonds become weaker. Protons without non-covalent interactions can move more freely and proton mobility increases. The highest proton mobility of HA was obtained at the 103 nm R_g and 46 nm R_h .

Intrinsic viscosity values are plotted against proton mobility. The maximum proton mobility was observed at 581,5 mL/g. As R_g and R_h , also intrinsic viscosity presented inverse correlation with proton mobility. Chain overlap point was found at 1233 mL/g where HA coils start to touch and overlap. Above and below this intrinsic viscosity, HA solutions have two different properties. Below the chain overlap point polymer coils move more freely, HA solution has fluid character and shows high proton mobility. However, above the value polymer coils interact with each other, solution gains highly viscous, gel like behavior and presents low proton mobility.

The parameters (R_g , R_h and intrinsic viscosity) in acidic conditions were not tested at different acid concentration, because HA loses its polyelectrolyte property, turns into acidic form and aggregates.

R_g/R_h values were plotted against proton mobility and the effect of conformation on the proton mobility was investigated. HA with worm like rod conformation had the highest proton mobility compared to gel, aggregated compact sphere and rigid rod conformations.

HA has the highest proton mobility in 0,5 M NaOH as a worm like rod conformation at 103 nm R_g , 46 nm R_h and 581,5 mL/g intrinsic viscosity.

With this study, it was possible to increase the proton mobility of high molecular weight HA from ~53% to ~100%. A practical application of this study is the determination of DS% of HA modified sample.

The degree of substitution for the benzylamine modified HA dissolved in water calculated as 23% and for the same sample dissolved in water with NaOD resulted 15%. These two different results are caused by different proton mobility of HA.

Performing DS% analysis with inhomogeneous proton mobility in the molecule produce big mistakes on the quantification. With this work, we are able to understand the causes of the low proton mobility and tune it as is required.

This study shows that proton mobility directly depends on the dimension of polymer in solution and the chain overlap point. Independent of any method which decrease the chain dimension of polymer (with enzyme or reagent), only the dimension of polymer must be under the chain overlap point in order to perform correct NMR measurement.

A similar principle can be applied to solve also those problems observed for other carbonhydrates such as alginate and chitosan proton mobility.

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APPENDICES

APPENDIX A: Reagent concentration versus proton mobility% graphs

APPENDIX B: R_g and R_h versus proton mobility% graphs

APPENDIX C: Intrinsic viscosity versus proton mobility% graphs



APPENDIX A

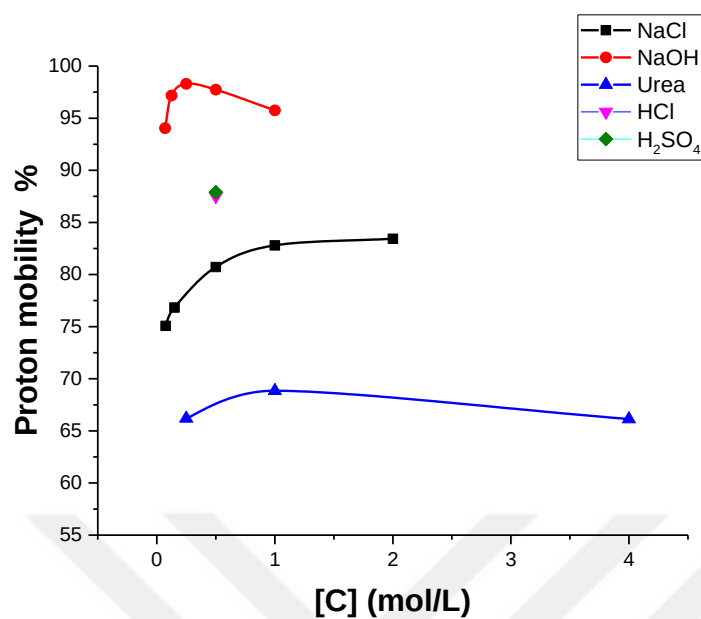


Figure A.1 : Percentage of proton mobility of sugar backbone hydrogens as a function of reagent concentration.

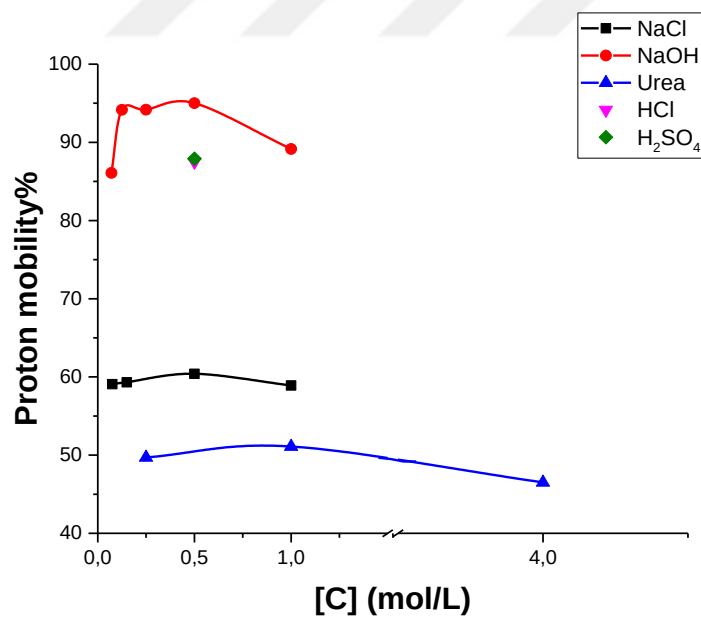


Figure A.2 : Percentage of proton mobility of anomeric hydrogens as a function of reagent concentration.

APPENDIX B

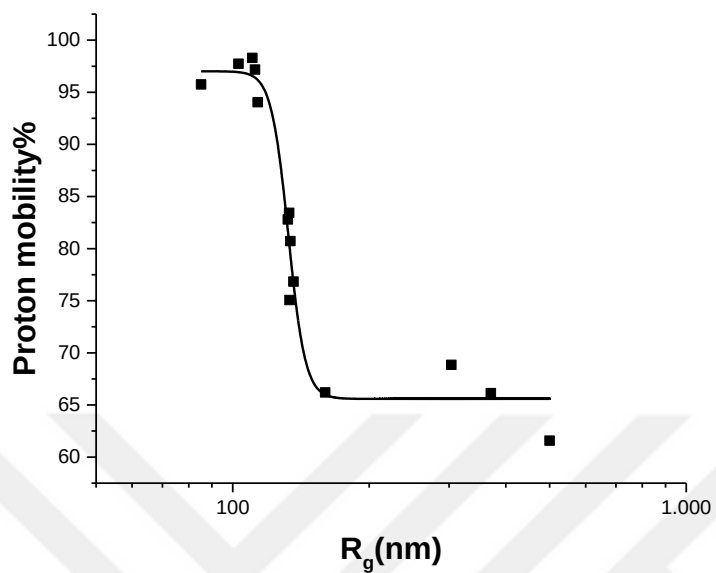


Figure B.1 : Relation between radius of gyration and proton mobility % of sugar backbone hydrogens.

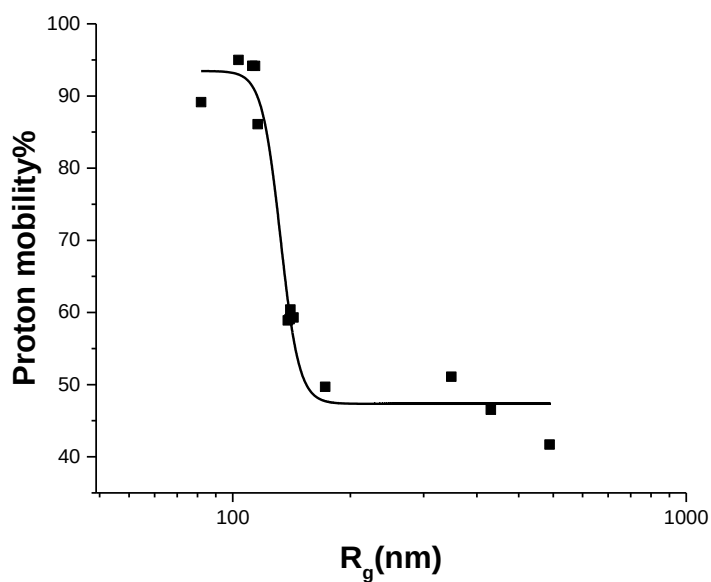


Figure B.2 : Relation between radius of gyration and proton mobility % of anomeric hydrogens.

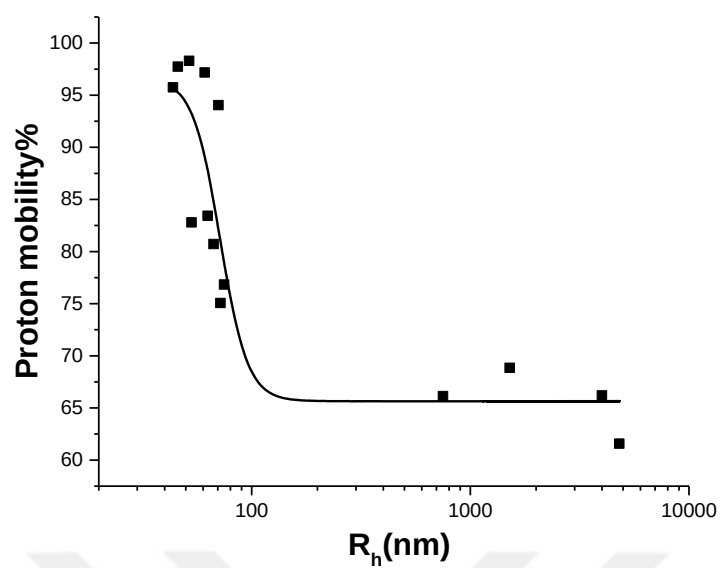


Figure B.3 : Relation between hydrodynamic radius and proton mobility % of sugar backbone hydrogens.

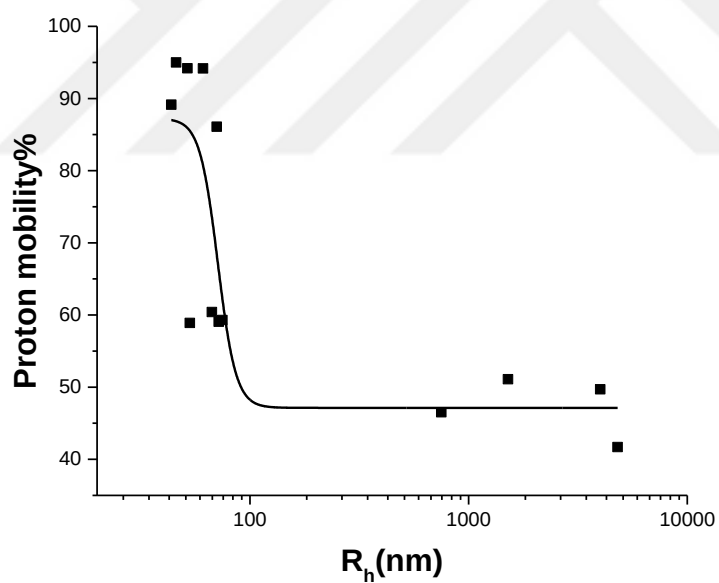


Figure B.4 : Relation between hydrodynamic radius and proton mobility % of anomeric hydrogens.

APPENDIX C

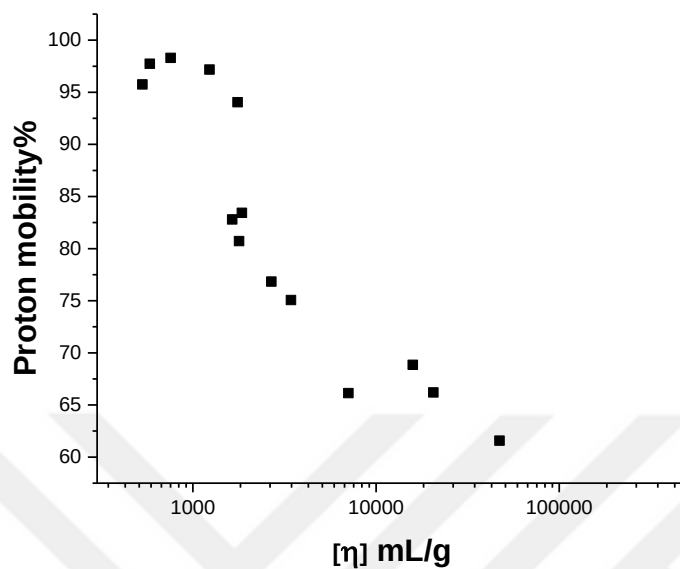


Figure C.1 : Relation between intrinsic viscosity and proton mobility % of sugar backbone hydrogens.

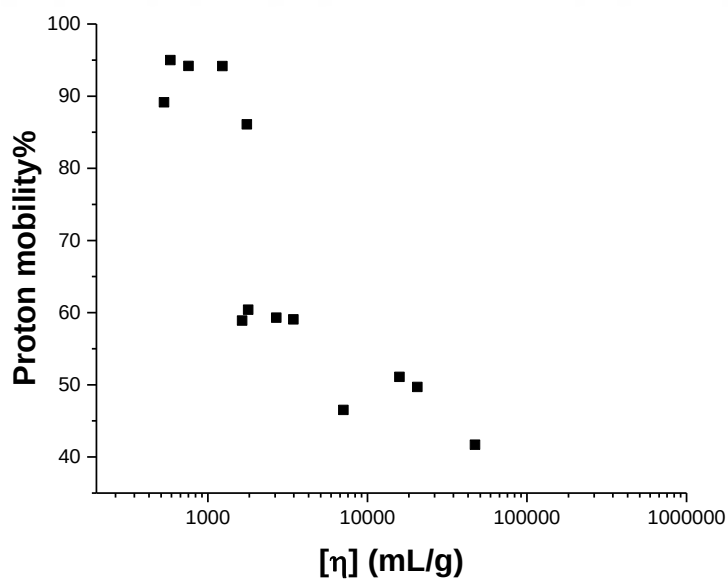


Figure C.2 : Relation between intrinsic viscosity and proton mobility % of anomeric hydrogens.



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