FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING TO PROBE THE DIFFUSION PROPERTIES IN HYALURONAN

Hélène DIEUSAERT

First master of pharmaceutical care

Academic year 2011-2012

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SIK
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Structure and Material Design

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June 4th, 2012

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Promoter

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Summary

In recent years, research has been performed on the diffusion of drugs out of pharmaceutical dosage forms. This information is important for the development of sustained release formulations which could release biologically active molecules during a longer time span. Hyaluronic acid (HA) is a biodegradable, biocompatible, mucoadhesive, non-toxic, non-inflammatory, non-immunogenic linear polysaccharide which has been used in several medical, pharmaceutical and cosmetic applications.

A fluorescence recovery after photobleaching (FRAP) study of different charged and sized probes was performed to shed light on the mass transport in HA under physiological conditions, together with an additional rheological characterization. Fluorescent probes (Na-fluorescein, 10 and 70 kDa FITC-dextran, amine-modified latex beads, carboxylate-modified latex beads, FITC-albumin and FITC-labeled dendrimers) were chosen to prepare 3 different hyaluronan samples in H₂O and in PBS (0,5%, 1% and 1,5%). FRAP-measurements were performed with a confocal laser scanning microscope and the diffusion coefficients were calculated in Matlab. The hyaluronan samples in H₂O were analyzed at a controlled temperature of 25°C and the ones in PBS were measured at 25°C and at 37°C to approach the human body conditions.

The absolute values of the diffusion coefficients of the probes increased with increasing the temperature from 25°C to 37°C due to the increased thermal motion of the molecules. The relative diffusion coefficients of the probes decreased with increasing concentrations of hyaluronan. This was caused by the increased obstruction of the movement of the molecules through the network. The Na-fluorescein molecules were too small to undergo interactions with the hyaluronan, even in higher concentrations. The FITC-dextran molecules are slowed down by increasing the concentrations of hyaluronic acid. Sticky interactions were determined for the FITC-labeled albumin probes and the FITC-labeled dendrimers.

Future research could focus on the diffusion characteristics of other positively charged fluorescent probes, the sample-preparation methods of the latex beads and the determination of the range of linear relationship between fluorescence intensity and FITC-dendrimer concentration.
Samenvatting

De laatste jaren is al veel onderzoek verricht naar de diffusie van geneesmiddelen uit farmaceutische preparaten. Deze informatie is belangrijk voor de ontwikkeling van preparaten die biologisch actieve stoffen zouden kunnen vrijstellen gedurende een langere periode. Hyaluronzuur is een biodegradeerbaar, biocompatibel, muco-adhesief, niet-toxisch, niet-inflammatoir, niet-immunogeen lineair polysaccharide dat al gebruikt werd voor medische, farmaceutische en cosmetische doeleinden.

Een fluorescence recovery after photobleaching (FRAP) - studie werd uitgevoerd met probes met verschillende landingen en groottes om meer te weten te komen over het massatransport in hyaluronan onder fysiologische omstandigheden. De stalen werden ook rheologisch gekarakteriseerd. Fluorescente probes (Na-fluoresceïne, 10 en 70 kDa FITC-dextraan, amine-gemodificeerde latex beads, carboxylaat-gemodificeerde latex beads, FITC-albumine and FITC-gelabelde dendrimeren) werden gebruikt om hyaluronan stalen te bereiden in H₂O en in PBS (0,5% , 1% en 1,5%). De FRAP-experimenten werden uitgevoerd met een confocale laser scanning microscoop en de diffusiecoëfficiënten werden berekend met behulp van een script in Matlab. De stalen in H₂O werden geanalyseerd bij een gestandaardiseerde temperatuur van 25°C en de experimenten met de stalen in PBS werden uitgevoerd bij 25°C en 37°C om de omstandigheden in het menselijk lichaam te benaderen.

De berekende diffusiecoëfficiënten van de probes verhoogden als de temperatuur steeg van 25°C naar 37°C door de verhoogde thermische beweging van de moleculen. De relatieve diffusiecoëfficiënten van de probes verminderden in stijgende concentraties hyaluronzuur door de vermeerderde obstructie van de beweging van de moleculen door het netwerk.

Wanneer een Matlab-script gebruikt werd dat rekening hield met de mogelijkheid dat interacties opgetreden hadden, werden interacties waargenomen tussen het hyaluronzuur en de FITC-gelabelde albumine probes en de FITC-gelabelde dendrimeren.

Verder onderzoek zou kunnen focussen op de diffusie karakteristieken van andere positief geladen fluorescente probes, de staalvoorbereiding van de latexbeads en de bepaling van de range waarin een linaire relatie geldt tussen de fluorescentie en de concentratie van de FITC-dendrimeren.
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<tr>
<td>ALB</td>
<td>Amino-modified latex beads</td>
</tr>
<tr>
<td>AOBS</td>
<td>Acousto-optic beam splitter</td>
</tr>
<tr>
<td>AOTF</td>
<td>Acousto-optical tunable filter</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CLB</td>
<td>Carboxylate-modified latex beads</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscope</td>
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<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid; Hyaluronan</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly amido amine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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1 INTRODUCTION

FRAP (Fluorescence Recovery After Photobleaching) was already invented in 1974 [1], but thanks to developments in the fields of microscopy and mathematical models, it has become a versatile tool for studying local diffusion properties. In recent years, new models have been developed to investigate the diffusion in food products, hydrogels, cell cultures and drug delivery systems [2]. It is important in a pharmaceutical context to be able to characterize at a molecular level the release of drugs out of dosage forms, the delivery and the uptake into a certain location in the body [3].

Hyaluronic acid is an unbranched glycosaminoglycan and is an important macromolecule in the human body. It maintains the elastoviscosity of the synovial fluid and is a main component of the extracellular matrix and is so involved in tissue repair, inflammation, cell migration and proliferation. Due to its biocompatibility, it is in the focus of intensive research on potential applications as drug delivery agent and in tissue-engineering [4].

FRAP-studies have already been performed on hyaluronic acid to investigate its properties [5,6,7]. This study was motivated by the question, if we can understand more about the diffusion dynamics and electrostatic probe (drug) – HA interactions. In other words, to test if the known drug-release times can be tracked down to simple electrostatically driven release mechanisms. In this master thesis project we wanted to characterize the diffusion of variously charged and sized fluorescent probes in entangled HA gels in H₂O and in physiological conditions by FRAP-measurements. Our chosen probes have different charges, hydrophilicity and sizes.

The introduction in this Chapter 1 is separated into a general introduction and five sections describing the theoretical background of this study. An overview of the applications of hyaluronic acid in a pharmaceutical and medical context and the theoretical background of the experiments is discussed. The objectives of the study are written down in Chapter 2. In Chapter 3 the used materials and methods are described, followed by a discussion of the results in Chapter 4 and 5. Conclusions are formulated in Chapter 6.
1.1 HYALURONIC ACID

Hyaluronic acid (HA) or hyaluronan was first purified from the viscous vitreous humor of bovine eyes by Karl Meyer and John Palmer in 1934 [8]. Hyaluronic acid refers to the presence of uronic acid in the polymer structure and ‘hyal’, the Greek word for glass. It is a linear unbranched glycosaminoglycan (GAG) polymer composed of repeating β(1,4)-N-acetylglycosamine – β(1,3)-D-glucuronic acid units. Unlike other members of the GAG family, like chondroitine sulfate and heparin, HA is not sulfated.

HA and its derivates form pseudo-plastic solutions that exhibit a shear-dependent viscosity and frequency-dependent elasticity [10].

It is found in particularly high concentrations in synovial fluid, the vitreous fluid of the eye, umbilical cords and chicken combs [9]. HA is a main component of the extra cellular matrix (ECM) and the glycocalyx and is naturally involved in tissue repair, cell migration and proliferation, inflammation and tumor growth and metastases [11]. It is synthesized by a class of integral membrane proteins called hyaluronan synthases and degraded by hyaluronidases.

1.1.1 Biological functions

The main function of hyaluronan is to lubricate the movable parts of the body such as joints and muscles. It is a good moisturizer due to its hydrophilic characteristics. It maintains the elastoviscosity of liquid connective tissues, controls tissue hydration and water transport and is involved in cell detachment, mitosis, migration, inflammation processes and tumor development and metastasis.

It is found in soft connective tissues of the skin, in the umbilical cord, synovial fluid, vitreous humor, lung, kidney, brain and muscle tissues.
It was reported that the physiological effects of hyaluronan may be very depending upon its average mass [12]. The degradation products of hyaluronan exhibit pro-angiogenic properties [9]. Hyaluronan is broken down during inflammation into lower molecular weight forms that interact with dendritic cells and induce their maturation [13]. They also induce the chemokine gene expression in alveolar macrophages that are important for the development and maintenance of the inflammatory response [14].

1.1.2 Pharmaceutical and medical use

Drug delivery from hyaluronan solutions is performed at several domains: in ophthalmic, pulmonary and nasal applications, by parenteral or topical way, in gene delivery and implantable drug delivery [15]. We can make use of HA in tissue engineering where three-dimensional scaffolds are built to deliver cells and bioactive substances to patients [4].

Due to the short in vivo lifetime and the low bioavailability, HA requires modifications to produce a material with the desired mechanical and functional properties. HA particles can be chemically adjusted and further modified by polymerization of anionic and cationic monomers to the HA surface [16].

Further research has to be done on the delivering of hydrogels without implantation, prolonging the release kinetics of drugs from hydrogels and expanding the nature of drugs which can be delivered using hydrogel based applications [17].

1.1.2.1 Dermal and Transdermal drug delivery

The stratum corneum is the outer layer of the skin and prevents the permeation of drug molecules into and across the skin [15]. HA has a beneficial effect on the dermal delivery of diclofenac, ibuprofen, clindamycin phosphate and cyclosporine. HA enhances the partitioning of diclofenac into the skin and its retention and localization in the epidermis and reduces its percutaneous absorption through the formation of a drug depot in the epidermis. Similar effects are reported for ibuprofen, clindamycin phosphate and cyclosporine.

1.1.2.2 Vaginal delivery

Vaginal delivery is considered as an important route for local and systemic drug administration due to its large surface area, good blood supply, high permeability and avoidance of the first-pass metabolism [18]. Traditional commercial preparations often
require multiple daily doses owing to the self-cleaning action of the vaginal tract. Solid or semi-solid mucoadhesive therapeutic systems have been developed that form hydrogels upon administration. Examples of possible mucoadhesive polymers are synthetic polyacrylates, chitosan, cellulose derivates, pectin and hyaluronic acid derivatives.

1.1.2.3 Ocular delivery
HA can be used in ocular delivery because it is not irritating and it has a high water-binding capacity. It is able to stabilize the tear film and relieve the pain and itching sensations that characterize dry-eye-syndrome [19]. Its viscosity, pseudoplastic behavior and mucoadhesive properties increase the ocular residence time [20].

![FIGURE 1.2: HYLO-COMOD®. Lubrication of dry eyes [21].](image)

1.1.2.4 Nasal delivery
Nasal delivery is examined as an alternative for systematic drug delivery because rapid absorption is possible due to the large surface area and the high blood flow and because first-pass elimination by the liver can be avoided. The disadvantage of poor bioavailability still needs to be overcome. The nasal absorption of drugs and proteins could be enhanced by the mucoadhesive properties of HA. This mucoadhesive behaviour can be increased by conjugation of HA with other bioadhesive polymers as chitosan [20].

1.1.2.5 Pulmonary Drug Delivery
Pulmonary drug delivery of proteins and peptides is considered as a way of provoking systemic therapeutic effects without repetitive injections [15]. HA was included in these inhalation formulations to enhance the absorption and achieve sustained release.

1.1.2.6 Parental delivery
HA has been useful for the development of slow-release formulations of recombinant therapeutic proteins or peptides. HA is a protein-friendly drug carrier due to its hydrophylic
nature that prevents proteins from denaturation [22]. Inside the body the gel precursors can crosslink physically due to the change in temperature and pH or chemically by Michael type addition and disulfide bond formation [20]. The relatively slow gelation process could cause a large amount of drugs to diffuse away and could lead to an overdose. To overcome this problem cholesteryl group-bearing pullulan (CHP) nanogels were used as drug reservoir and were physically dispersed in a three-dimensional network of chemically cross-linked HA [22]. Another solution is to make use of the electrostatic interaction between the negatively charged carboxylate groups of HA and the positive surface charge of Fe$_2$O$_3$ nanoparticles.

1.1.2.7 Intra-articular delivery
Intra-articular drug delivery is an attractive treatment approach for osteoarthritis since this degenerative joint disease is now mainly treated with various glucocorticoid and hyaluronic acid formulations which are often not disease modifying, provide only short-term pain relief and have notable side effects [23].

![FIGURE 1.3: Comparison of systematic cross sections of a healthy joint (left) and a joint affected by osteoarthritis (right) [23]. Proteoglycan loss leads to damage of the collagen network. OA is characterized by progressive loss or articular cartilage, subchondral bone sclerosis, osteophyte formation, changes in the synovial membrane and increased amount of synovial fluid with reduced viscosity.](image)

1.1.2.8 Implantable delivery
Research has been done on the development of an implantable delivery device that would be able to guarantee long term delivery of protein drugs [15]. A mixture of chitosan with HA
was used. This complex swells due to water penetration and the zero order release of drugs is dependent on the composition of the formulation.

1.1.2.9 Liposomal Drug Delivery
A liposome is a vesicle composed of a lipid bilayer of natural phospholipids that can be used as a vehicle for administration of nutrients and drugs (Figure 1.4). They may contain lipid chains with surfactant properties and surface ligands. Hydrophilic drugs can be encapsulated in the aqueous solution inside the liposome and hydrophobic drugs can be dissolved into the lipophilic membrane. The liposome delivers drugs by fusion with (cell)membranes. This way of drug delivery would increase drug solubility, prolong the half-lives of drugs in the body and protect the drugs [15].

![FIGURE 1.4: Liposome with drugs inside](image)

Liposomes composed of lipids with attached hyaluronic acid showed improved function and stability, could be targeted to specific cell-lines, acted as sustained-release carriers of drugs and provided comparable or better efficiency of drug encapsulation.

1.1.2.10 Gene therapy
Nowadays most gene therapy studies are concentrated on inhibition of oncogene expression. The efficiency of gene therapy is limited by poor intracellular uptake, insufficient endosomal escape of genes and rapid enzymatic degradation of DNA or RNA. Various cationic polymers, HA, polypeptides and lipids have been proposed as carriers for gene delivery [20].

1.1.2.11 Oncologic use
HA shows high affinity to CD44 and RHAMM which are over-expressed in some tumor cells and is therefore used for targeting specific intracellular delivery of genes or anticancer drugs
[20]. Coupling to HA can also provide advantages in drug solubilization, stabilization, localization and controlled release.

**FIGURE 1.5: Target-mediated endocytosis delivery** [20]

1.1.2.12 Medical use

Hyaluronic acid and derivates can be used therapeutically in different domains: viscosurgery, viscoaugmentation, viscoseparation, viscosupplementation, viscoprotection, drug delivery and tissue engineering [25]. Esterified HA is also used to prevent bacterial adhesion to dental implants, intraocular lenses and catheters [9].

- **Hyaluronan** was used for the first time in a medical context as a vitreous substitution during **eye surgery** in the late 1950s [25].
- **Viscoaugmentation** with hyaluronic acid gels is used to fill facial wrinkles and depressed scars and to treat glottal insufficiency.
- **Hyaluronan solutions** separate tissue surfaces that slide along each other and reduce postoperative adhesion after abdominal or orthopedic surgery [9].
- **Viscosupplementation** with HA and HA derivates is a safe local treatment for osteoarthritis (OA). It relieves the pain, restores the protective viscoelasticity of the synovial hyaluronan and improves mobility [7,23].
- **Tissue engineering** is an upcoming alternative for dangerous transplantations [4]. The goal is to restore, maintain or improve tissue function through three-dimensional scaffolds that deliver cells or bioactive substances. Hyaluronic acid hydrogels are an ideal material for tissue engineering thanks to its biocompatibility and biodegradability but modification with adhesion-mediating peptides is required.
1.1.3 Physicochemical properties

1.1.3.1 Structure

D-glucuronic acid and D-N-Acetyl-glucosamine are linked through β(1,4) and β(1,3) glycosidic bonds as can be seen in Figure 1.1 [9]. Hyaluronan synthases synthesize linear polymers with up to 10,000 or more disacharide units. A stretched hyaluronan molecule can reach up to 10 µm. The bulky hydroxyl and carboxylate groups are in the sterically favorable equatorial positions and form a polar hydrophilic face. HA-strains in dilute solutions adopt semi-flexible random-coil configurations with an apparent persistence length of 5nm and occupy a large hydrated volume (Figure 1.6) [26]. In concentrated solutions, stiffened random coils will show regions of domain-overlap and entanglement but they do not strongly self-associate [7]. HA-chains with a molecular weight of 3x10⁶ Da start to overlap if the HA concentration exceeds 0,6 mg/mL [5]. Chain-chain associations due to hydrophobic interactions are favored with increasing concentrations. The stiffening can at least partly be explained by the forming of hydrogen bonds between adjacent saccharides and by electrostatic repulsion between carboxyl groups. Ordered conformations and strong self-association can exist under specific conditions. They do not form gels.

FIGURE 1.6: Conformations of hyaluronan observed in TMAFM (tapping mode atomic force microscopy) height images of high molecular weight rooster comb hylan A. (left) coiled conformation deposited on freshly cleaved mica from 5 µg/mL solution in 10 mM MgCl₂ (scale =500nm). (middle) Condensed conformation deposited from 500µg/mL solution in 0,15 M NaCl (scale =250nm). (right) Intermolecular aggregation of intramolecularily condensed chains of HA deposited on freshly cleaved mica from 500µg/mL solution in 0,15 M NaCl (scale=250nm) [26].
1.1.3.2 Modified hyaluronan

The characteristics of HA solutions can also be changed by inserting modifications. Chemical modification is often achieved by targeting the hydroxyl groups on the sugar rings or the carboxylic acid groups present on the glucuronic acid moieties [4]. Modifications should be performed under mild conditions of pH and temperature, and low-degree functionalization should be enabled in order to maintain the properties of native HA.

HA gels can be formed by covalent cross-linking or with the use of homo-bifunctional cross-linkers. If the solute concentration is sufficiently high, the system passes from a dilute to a semi-dilute system and an effect similar to gelling occurs. As the concentration exceeds the critical overlap concentration \( c^* \), separate polymer chains start entangling. \( c^* \) can be seen as a sudden change in the diffusion coefficient if the hyaluronan concentration is increased [27].

Esterified HA is used to prevent bacterial adhesion to dental implants, intraocular lenses and catheters [9].

HYLANS

Hylans are a group of chemically modified hyaluronans and they include hylan fluids, gels, membranes and microparticles [19]. These higher molecular weight forms are produced by chemically crosslinking the HA-chains without affecting the carboxylic acid or the N-acetyl groups. Formaldehyde crosslinking forms soluble hylan while vinylsulfone crosslinking gives raise to infinite molecular networks that form gels, membranes or microparticles. Soluble hylan has a higher elasticity and viscosity at low frequencies and higher shear rates than hyaluronan. Hylan fluid is a water-soluble, polyanionic, hydrophilic hyaluronan derivative which is the basis for various release systems due to its excellent biocompatibility. Hylan gels have a at all frequencies a greater elasticity and have at lower shear rates a greater viscosity than soluble hylan.

Hylans can be used as bifunctional delivery systems that have a function in the controlled-release of drugs as well as a physical function.

It is possible to do chemical modifications as increasing the net negative charge without losing the biocompatibility. By varying the crosslink density, the release kinetics for specific
drugs may be altered. Hylan biomaterials can be combined with coatings or co-polymers. The release-kinetics are usually diffusion-driven but hyal systems can be designed to deliver cationic drugs with reduced large initial burst release of the drugs and with continued release over a longer period of time[19].

1.1.3.3 Rheology

The viscosity of HA solutions is dependent upon crosslinking, average molecular weight of HA, entanglement, pH, solvent, chemical modifications and the shear rate at which the measurements are done, while the absolute concentration seems to be less important [20]. HA solutions exhibit viscoelastic properties due to its polymeric (high molecular weight, double helical conformation) and polyelectrolyte (electrostatic forces and intramolecular H-bonds) properties [7]. Since relatively small forces (depending on their impact frequency) can cause much larger elastic deformations then in liquid rubber, the polymer belongs to the class of the ‘super-elastic liquids’ [25]. HA and its derivates form pseudo-plastic solutions that exhibit a shear-dependent viscosity and frequency-dependent elasticity. The relation between the shear stress and the shear rate is not constant and therefore one constant viscosity coefficient cannot be defined (Figure 1.7).

![Figure 1.7](image_url)

**FIGURE 1.7:** Time-independent rheology of fluids presented as shear stress as a function of shear rate. (- Newtonian fluid, - Dilatant (shear thickening) fluid, - Pseudoplastic (shear thinning) fluid)[28].

Rheological data of HA in reference to the effect of ionic strength, hydrogen ion concentration and polymer concentration are nicely described in [10]. A good introduction to rheology can be found in [29].
1.1.3.4 Effect of pH and counter-ions

EFFECT OF PH

Alkali affect HA chain stiffness profoundly: if NaOH is added, large increases in self-diffusion coefficients occur due to disruptions of the hydrogen bonds between adjacent saccharides [6].

COUNTERIONS

Electrostatic interactions are sensitive to counter-ions. Investigation of the effect of increasing electrolyte concentration on HA solutions showed that the self-diffusion coefficient of HA was very low in the absence of any electrolyte, but increased dramatically with small increases in NaCl concentration. This can be explained by increased electrostatic shielding resulting in poly-anion coil-contraction. Since this was complete at 100mM NaCl, the contribution of ionic strength and pH to molecular stiffness is suggested to be small [6]. Investigation of the effects of different counter-ions showed that Ca$^{2+}$ caused a significant increase in self-diffusion of HA compared with Na$^+$, with less increase with Mn$^{2+}$ and Mg$^{2+}$ [7]. The contraction of the HA-domain in calcium solutions suggested that Ca$^{2+}$ increased the flexibility of the chain by promoting a greater range of movement at each glycosidic bond. This is possible by altering the coordination of water molecules with HA chains, causing the interruption of hydrogen-bonds between adjacent sugars [30]. The solution properties at higher concentrations are related to the hydrodynamic volumes of single chains in the same solvent. The bulk properties and mobility of individual HA-molecules is temperature-independent between 20 and 70°C.

1.2 DIFFUSION

Diffusion is the random movement of particles in a medium. In 1827 Robert Brown observed in his microscope that little particles ejected by pollen grains suspended in water executed a jittery motion. He was able to rule out that the motion was life-related by repeating this experiment with particles of inorganic matter. The random motion of colloidal particles is caused by the chaotic thermal motion of fluid or gas molecules and is known as ‘Brownian
motion or ‘random walk model’. It is assumed that for a series of steps each step is uncorrelated with the previous one. It yields a Gaussian probability distribution for long diffusion times and a large number of particles [31].

Diffusion is controlled by the second law of thermodynamics. This law states that everything in the universe strives towards equilibrium. The entropy or disorder in an isolated system always increases or remains constant. Molecules in an area with high concentration will move to an area with a lower concentration with a magnitude that is proportional to the concentration gradient until there is no net diffusion anymore and the system has reached equilibrium. The diffusion equation (Eq 2.1) describes density fluctuations in a material that is undergoing diffusion.

\[
\frac{dc}{dt} = -D \frac{d^2c}{dx^2}
\]  

(Eq 2.1)

- \( t \) = time [s]
- \( D \) = diffusion coefficient \([\text{m}^2/\text{s}]\)
- \( C \) = concentration \([\text{mol/m}^3]\)
- \( x \) = position [m]

Colliding molecules transfer kinetic energy and change their direction and velocity.

The easiest way to express the probe size is to give the molecular weight or the radius of the particle. The Stokes-Einstein equation (Eq 2.2) is the equation used to derive the diffusion coefficient of a small spherical particle undergoing Brownian motion in a viscous fluid at uniform temperature

\[
D_0 = \frac{kT}{6\pi n Rh}
\]  

(Eq 2.2)

- \( D_0 \) = Diffusion coefficient \([\text{m}^2/\text{s}]\)
- \( k \) = Boltzmann’s constant = \(1.3806503 \times 10^{-23}\) \(\text{m}^2\text{kg s}^{-2}\text{K}^{-1}\)
- \( T \) = Temperature [K]
- \( n \) = Viscosity of the solvent [Pa.s]
- \( Rh \) = hydrodynamic radius [m]
Knowing the diffusion coefficient, the Stokes-Einstein equation can also be used to estimate the hydrodynamic radius of the regarding molecules.

Research has been performed on the effects of the probe size, the gel concentration, the viscosity, ageing and enzymatic treatment on the diffusion rate. A bigger probe will diffuse more slowly than a smaller probe as can be seen in Figure 1.8.

![FIGURE 1.8: Diffusion coefficients of DNA molecules in PBS as a function of their size in basepairs. Solid squares indicate supercoiled DNA, open squares indicate linear DNA. The triangles and lines represent values from the literature. Image reprinted from [32].](image1.png)

When the concentration of the gel increases, the gel becomes denser and more possibilities arise for crosslinking or entanglement. Ageing causes a decrease in pore size over time due to relaxation of the network as is illustrated in Figure 1.9. Both the rotational and the translational diffusion can be lowered [33].

![FIGURE 1.9: Large FITC-labeled BSA (bovine serum albumin) probes can only rotate and become completely hindered in time, the lateral diffusion of FITC-HRP (horseradish peroxidase) will decrease with increasing ageing time while the small FITC molecules can keep on moving freely around [33].](image2.png)
1.3 FLUORESCENCE

Fluorescence is the ability of a molecule to absorb electromagnetic radiation of a particular wavelength and subsequently reemit it at a lower wavelength after a short time interval ($10^{-9}$ s). Absorption and emission of a photon can only happen at certain specific wavelengths, called the absorption and emission band or spectrum. The emission-wavelengths are longer than the absorption-wavelengths due to loss of energy by interactions with the environment. Each fluorescent molecule can undergo this process several times before it becomes bleached by oxidation of the excited molecule. Since most samples are not auto-fluorescent, an external fluorescent probe has to be added to visualize the sample.

The preferable fluorophore used in a certain FRAP experiment depends on the available excitation source, the hydrophilic properties of the medium and the chemical ways available to attach it to the molecule of interest [34]. One of the most used hydrophilic fluorophores is fluoresceine isothiocyanate (FITC). It can easily be attached to proteins and polysaccharides.

The fluorescent probes used in this study are described in Section 4 Material and Methods.

1.4 FRAP

We used confocal fluorescence recovery after photobleaching during this project. This is a method to study the mobility of fluorescent probes in different materials. It is required that the materials are stainable and translucent.

In confocal-FRAP, the intensity in a plane is measured with a scanning laser beam and a detector. The bleaching has to be strong enough to prevent probes from other perpendicular planes to reach the ROI.

A typical FRAP-measurement is shown in Figure 1.10. To perform FRAP-measurements, the background has to be stained with a fluorescent marker. Thereafter a certain region of interest (ROI) in the sample is bleached with a strong laser beam. This ROI can be of different geometries e.g. a circle, a square, a line, a cell part or a fringe pattern [2]. Photobleaching means that some of the fluorescent markers lose their fluorescent properties. The intensity of the fluorescence in the ROI decreases ($F(0)$), but recovers again due to diffusion from nearby not-bleached regions. The bleaching is assumed to be irreversible.
FIGURE 1.10: Schematic representation of a FRAP-experiment. At \( t < 0 \) the fluorescent plane is scanned and the intensity is measured \( (F_0) \). The ROI (Region of Interest) is bleached by a strong laser beam at \( t = 0 \) and the fluorescence intensity drops down to \( F_0 \). Molecules diffuse out and in of the ROI and the fluorescence intensity recovers. At the end of the experiment the fluorescence intensity recovered up to \( F_\infty \).\[3\]

By recording the fluorescence intensity after photobleaching for a time span until the recovery plateaued, the computer software can calculate the apparent translational diffusion coefficient \( D \). The framework stated in \[35\] was used to analyze the data and calculate the diffusion coefficient. The Stokes-Einstein equation (Eq 2.2) can then be used to calculate the hydrodynamic radii. A quick recovery of the intensity means that the probe can diffuse fast through the medium. Large probes, ionic interactions or dense structures can cause a slower recovery. Sometimes the fluorescence intensity doesn’t recover totally \( (F_\infty) \). This can be due to an immobile fraction of probes which are bleached, but that cannot be replaced by migrating fresh fluorescent probes \[3\].

Confocal-FRAP is an equilibrium method that is carried out in the absence of flow and shear forces and with no concentration gradient.

1.4.1 Applications of FRAP
FRAP was originally developed to study molecular mobility in biological samples \[36\]. It allows us to perform localized diffusion experiments and to study mobility and interactions in small \((10-100\mu L)\) intact samples \[34\]. This technique has been used in different areas: gels, cells, solutions, emulsions and lipid membranes \[2\].

FRAP can be used to study the mobility in the cytoplasm of cells (influenced by obstacles like the cytoskeleton) or in the interstitial spaces of tissues, which is important for the delivery of
substances from the blood to the cells. Cellular binding interactions slow down the recovery after photobleaching. The next step is to determine the respective contributions of diffusion and binding to the recovery curve (Figure 1.11). When the diffusion time is much shorter than the time to begin binding, the initial fast diffusive phase is followed by a much slower recovery of fluorescence due to the bound molecules, and diffusion-uncoupled FRAP recovery takes place. If a molecule is bound before it can diffuse far, the diffusion time is comparable to the time for a molecule to associate with a binding site and diffusion-coupled FRAP-recovery takes place. In such cases, the time for complete recovery is additionally determined by a pseudo-association rate (related to the time a molecule diffuses freely between 2 interactions) and the off-rate (related to the time the probe is bound).

**FIGURE 1.11: Diffusion coupled and uncoupled FRAP** [36]. • Fluorescent molecule, • bleached molecule, □ Binding site

FRAP is a versatile tool in studying drug mobility and interactions not only in hydrogel dosage forms, but also in conventional formulations that become a gel upon hydration.

If FRAP is used for measuring the lateral diffusion coefficient in cell membranes, the diffusion coefficients vary due to immobile fractions, directed motions due to active transport and constrained random motion due to interactions [34]. FRAP can be used in binding studies and immunological assays by estimating the fraction of immobile molecules. It is also possible to perform flow velocity measurements.
Different FRAP variants exist such as Total Internal Reflection microscopy-FRAP, polarized FRAP, continuous photobleaching and scanning microphotolysis [34].

1.4.2 Future applications in research
FRAP measurements could be used in the future in the research on the mobility of drugs in macro- or microscopic pharmaceutical dosage forms, mobility and binding of anti-tumor drugs in tumor tissue, intracellular transport of gene complexes and mobility of drugs in membranes [34].

1.5 CONFOCAL LASER SCANNING MICROSCOPY
A Confocal Laser Scanning Microscope (CLSM) was used for the FRAP-measurements. A CLSM can give us information about an optical section in the sample. It is possible to construct non-invasive 3D images by piling up the images of parallel focal planes.

The principle for CLSM was already invented in 1955 by Marvin Minsky, but it took 20 more years to apply it to biological specimens [2]. In Figure 1.12 we can see that a CLSM consists of a laser, AOTF (Acousto-Optical Beam Splitter), illumination aperture, beam splitter, scanning mirrors, objective and a specimen on a stage [37].

![Figure 1.12: The main components of a Confocal Laser Scanning Microscope](image-url)
2 OBJECTIVES

In recent years, a lot of research has been performed on the diffusion of drugs out of pharmaceutical dosage forms. This information is important for the development of sustained release formulations. Up to now, it is often necessary to take one or more pills a day if constant plasma concentrations in the therapeutic window are required. Slow-release formulations could also improve the patient compliance.

Hyaluronic acid is a non-immunogenic, bio-degradable polysaccharide, which makes it promising for pharmaceutical and medical applications. It has negatively charged carboxylic acid groups.

We aimed to investigate the ionic characteristics of the diffusion of probes through a hyaluronic acid matrix by FRAP-measurements. Especially the influence of positively charged probes (drugs) on the mass transport in HA is unclear. We first wanted to make sure that the probes motion would get restricted and would slow down with increasing concentrations of hyaluronan. Thereafter we tried to investigate whether there would be a difference in the diffusion coefficient if the probes were diluted in PBS instead of in H$_2$O. We were also interested in the relationship between the characteristics of the probes (hydrophobicity, charge, molecular weight,...) and their behavior in the hyaluronan solutions. We expected that if the probes would interact with the negatively charged groups, the diffusion-rate would decrease and a special Matlab script would be used to calculate the characteristics of these binding interactions.

A first range of tests was performed on hyaluronic acid dissolved in distilled and ultrafiltrated water in 3 different concentrations (0,5% 1% 1,5%). Probes with a negative, positive and neutral charge were chosen. FRAP-experiments were conducted with a confocal laser scanning microscope and the diffusion coefficients were calculated in Matlab. A second range of tests was implemented on hyaluronan solutions with the same concentration in PBS-buffer to approach the human body fluid conditions. A temperature control stage was used during the FRAP-measurement in H$_2$O at 25°C and in PBS at 25°C and 37°C. A further sample characterization is important to decrease the chance that confounding occurs. This was achieved by additional pH-measurements and rheological tests.
3 MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Hyaluronic Acid
The used hyaluronic acid was kindly provided by Bohus Biotech and the approximate molecular weight was $4,2 \times 10^6$ Da. More characteristics and applications of HA can be found in Section 1.1.

![FIGURE 3.1: Hyaluronic acid.](image)

3.1.2 PBS
PBS buffer is a water-based salty solution in concentrations similar to those in the human body. It is isotonic and non-toxic to cells. 1 tablet (Sigma Phosphate buffered saline tablets) was dissolved into 200 mL demineralized water (0,01M phosphate buffer, 0,0027M KCl, 0,137M NaCl, PH 7,4 at 25°C) and stored in the fridge.

3.1.3 Fluorophores

3.1.3.1 Na-Fluoresceïn
Na-Fluorescein is an orange-brown powder. It is negatively charged in aqueous solutions (Figure 3.2).

![FIGURE 3.2: Na- fluoresceïn [38].](image)
Na-fluorescein has an excitation maximum of 494 nm that closely matches the 488 nm spectral line of the argon-ion laser used in confocal laser scanning microscopy. The emission wavelength is 514 nm. Fluorescein has a good fluorescence quantum yield but it has a relatively high rate of photobleaching.

The fluorescent properties are significantly reduced under pH 7 as can be seen in Figure 3.3.

![Figure 3.3: pH-dependent absorption (A) and emission spectra (B) of fluorescein [39].](image)

### 3.1.3.2 FITC-dextran

Dextran is a complex β(1,6)glucose polymer with branches beginning from α (1,3) linkages. It is water-soluble, biocompatible and biodegradable.

Fluoresceinisothiocyanato-dextran is a dextran polymer linked with fluorescein moieties by thiocarbamoyl linkages as can be seen in Figure 3.4. FITC-dextran is uncharged or slightly negative in solutions with pH 6.8 to 8.5.

![Figure 3.4: FITC-dextran. * The site of attachment is supposed to be randomly associated with any free hydroxyl group [40].](image)
FITC loses fluorescence when exposed to light at 495 nm and bleaches quite quickly at pH less than 7.0 with or without light exposure.

### 3.1.3.3 Carboxylate-modified latex beads (CLB)

These fluorescent probes are polystyrene latex beads with carboxylate-modified surface groups. Polystyrene is a linear carbohydrate with benzene groups attached to every second carbon atom as can be seen in Figure 3.5.

![Figure 3.5: Polystyrene](image)

The producer reported a mean diameter of 0.02-0.04 µm. The excitation wavelength is around 470 nm and emission wavelength is 505 nm. They have a negative surface charge density \( \sigma \) of 0.030-0.090 \( \mu \text{C/cm}^2 \) (Equation 3.1).

___

(Eq 3.1)

- \( \sigma \) = Surface charge density \( [\mu \text{C/cm}^2] \)
- \( E_t \) = Number of micro-equivalents acid or base used to neutralize 1g of polymer in titration
- \( \text{SSA} \) = Specific Surface Area \( [\text{cm}^2 / \text{g}] \) = Total surface area of all the polymer microspheres in 1g
- \( F \) = Faraday Constant = 96,485 C/equivalent

---
3.1.3.4 Amine-modified latex beads (ALB)

These probes are amine-modified polystyrene, fluorescent orange beads. Their excitation maximum is around 520 nm and the maximum value of the emission spectrum is 540 nm [42]. Their mean particle size is around 0.1 µm. In aqueous conditions, the amine groups are protonated and give the probes an overall positive charge.

3.1.3.5 FITC-Albumin

Albumin is an unglycosylated water soluble protein found in blood plasma and egg white with a molecular weight of 66 kDa. The main function is the regulation of the colloidal osmotic pressure of the blood but it also acts as a plasma carrier for hydrophobic steroid hormones and fatty acids due to its hydrophobic cavities. The albumin (Sigma-Aldrich, A9771, St. Louis, MO) we used was bovine albumin labeled with 7 to 12 mol FITC per mol albumin, bound through the epsilon amino group of the lysine amino acid residues. The excitation maximum is 495 nm and the emission maximum is 520 nm. Albumin is negatively charged when dissolved in water at pH 7.4.

3.1.3.6 FITC-labeled dendrimers

Poly AMido AMine (PAMAM) dendrimers consist of an alkyl-diamine core and tertiary amine branches. Each subsequent step in the manufacturing process represents a new generation and doubles the number of reactive surface sites. They have a narrow weight distribution, a specific size and shape, a high degree of molecular uniformity and a highly-functionalized terminal surface.

![FIGURE 3.6: Generation 2 PAMAM dendrimer [43].](image-url)
The used dendrimers were generation 2 dendrimers with an ethylenediamine core and an amine surface (Starpharma Holdings Limited, Melbourne, Australia). They are cationic at pH 7,4.

They were labeled with FITC (Sigma-Aldrich, St. Louis, MO) in the lab. The following procedure was used [44]: 0,5 mL of the PAMAM dendrimers were dissolved in 9,5 mL PBS. 4 mg FITC (Sigma-Aldrich, F3651) was dissolved in 1 mL acetone and added to the dendrimer solution after 2 hours. The molar ratio was 1,23 mol FITC per mol dendrimer. This solution was stirred overnight at room temperature. The next morning the fluorescent labeled dendrimers were dialyzed during 22 hours against distilled water using dialysis membranes of 0,5-1 kDa MWCO (Float-a-lyzer G2, Spectrum laboratories, Inc., Rancho Dominguez, CA). The distilled and ultrafiltrated water was replaced 4 times.

3.2 EXPERIMENTS

3.2.1 Sample preparation

Hyaluronic acid fibres (HA) (Bohus Biotech, Strömstad) were weighed on a scale (Mettler Toledo XS204) and put into a cup. A certain amount of diluted probe solution was added to obtain the desired concentration. A volume of 2 mL per sample was prepared to provide enough gel for two aluminum cups. The samples were stored at 4°C in a fridge to prevent the breakdown of HA at room temperature. The samples were stuck on a mechanical stirrer (Heidolph) at the lowest possible shaking speed to homogenize them. An experiment was done to test how long the HA-solution should be stored before measurements could be
performed. The manufacturer mentioned that a waiting time up to 72 hours could be needed. On 3 consecutive days, one 1% HA-sample was prepared and stored on a mechanical stirrer at 4°C in a fridge. The diffusion coefficients were measured on the fourth day. The diffusion coefficients of the 1 day old 1% hyaluronan solution were very variable, probably due to a too short homogenization time on the shaker. It was concluded that two days were enough to dissolve the fibers completely and to obtain a homogenous sample. The samples were taken out of the fridge 2 hours before the measurements were performed to let them slowly reach room temperature.

8 µL of the probes solution was put with a micropipette in a secure seal between 2 cover glasses (Figure 3.8).

**FIGURE 3.8:** 8 µL 100 ppm fluorescein in a secure seal between 2 cover glasses.

The hyaluronan solutions were put in an aluminum cup covered by a cover glass, and stored in aluminum foil to protect the probes from bleaching by sunlight (Figure 3.9).

**FIGURE 3.9:** Hyaluronan viscous solutions in an aluminum cup covered by a cover glass.
3.2.2 Experimental methods

The following tests were performed to characterize the samples.

3.2.2.1 pH-measurements

The pH was tested (Metrohm 632 pH-meter) because the fluorescence intensity of some probes is depending on the pH of the solvent (Figure 3.10).

![FIGURE 3.10: The pH-meter.](image)

3.2.2.2 Rheology

The visco-elasticity was tested with a rheometer (AresG2, TA Instruments) to additionally characterize the sample’s mechanical properties (Figure 3.11). Frequency sweep tests at 25°C and 37°C in a range of 0.01 to 15 Hz were performed at a constant strain of 1%, which was probed to be in the linear elastic region.

![FIGURE 3.11: The rheometer.](image)

3.2.3 FRAP-measurements

A Leica SP2 AOBS (Heidelberg, Germany) (Figure 3.12) was used with a 20x, 0,5 NA water objective. The following settings were chosen: 256x256 pixels and a zoom factor 4 (with a zoom-in during bleaching) and 800 Hz, yielding a pixel size of 0,73 µm. An image depth of 12 Bit was chosen. It was possible to make two images per second. The beam expander was set
to 1, which lowered the effective NA to approximately 0.35 and yielded a slightly better bleaching and a more cylindrical bleaching. A spherical ROI with a diameter of 30 µm was used.

FIGURE 3.12: The Leica SP2 AOBS, confocal laser scanning microscope with left the temperature control stage.

The diffusion coefficient was measured in the aluminum cup at 30, 50 and 100 µm depth under the upper surface. At a depth of 100 µm, the fluorescence intensity and the diffusion constant was decreased in comparison to the other measurements, probably due to filtering effects. It was decided to bleach at a depth of 50-60 µm under the surface in all the following experiments.

A temperature stage (MDS 600, Linkam) was used during the measurements to standardize the room temperature at 25°C, which lowered the standard deviations on the measurements.

FRAP-measurements were analyzed in MATLAB, running a script utilizing the statistical most-likelihood environment to calculate the diffusion coefficients. This framework is described in [35].

3.2.3.1 Concentration-dependent experiment

The goal of this experiment was to find out if the diffusion coefficients of different probes would change and how much it would change with increasing concentrations of HA.

The probes were diluted in destilled and ultrafiltrated water (Barnstead NANOpure Thermoscientific, Vantaa). The following probe concentrations (Table 3.1) were chosen in order to get enough fluorescence intensity to use the CLSM and to still stay in the linear range of the relationship between fluorescence intensity and fluorochrome concentration.
TABLE 3.1: Concentration and data of the used probes.

<table>
<thead>
<tr>
<th>PROBE</th>
<th>LOT#</th>
<th>COMPANY</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-fluorescein</td>
<td>456103/1</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
<td>100 ppm</td>
</tr>
<tr>
<td>10 kDa FITC-dextran</td>
<td>819564</td>
<td>Invitrogen, Eugene, Oregon, USA</td>
<td>200 ppm</td>
</tr>
<tr>
<td>70 kDa FITC-dextran</td>
<td>56318A</td>
<td>Invitrogen, Eugene, Oregon, USA</td>
<td>200 ppm</td>
</tr>
<tr>
<td>CLB (L5155)</td>
<td>012K1566</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
<td>500 µL in 9.5 mL H₂O / PBS</td>
</tr>
<tr>
<td>ALB (L9904)</td>
<td>MKBJ1904</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
<td>500 µL in 9.5 mL H₂O / PBS</td>
</tr>
<tr>
<td>PAMAM Dendrimer</td>
<td>3230371</td>
<td>Dendritic Nanotechnologies, Inc, Michigan, USA</td>
<td>1 % solution diluted 1:20</td>
</tr>
<tr>
<td>FITC (F3651)</td>
<td>101M5307V</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
<td></td>
</tr>
<tr>
<td>FITC-albumin (A9771)</td>
<td>080M7400</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
<td>400 ppm</td>
</tr>
</tbody>
</table>

A 0.5%, 1% and 1.5% Hyaluronan solution in diluted probe solution were prepared two days in advance and stored on a mechanical shaker at 4°C in a fridge. In a second range of tests, the probes were dissolved in PBS solution to approach the human body fluid. The same concentrations of hyaluronan solutions were prepared. FRAP-measurements were performed at 25°C and 37°C.
4 RESULTS AND DISCUSSION

4.1 Characterization of the sample

4.1.1 pH-measurements

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH (100 ppm NF in H2O)</th>
<th>pH (10 kDa FITC-dextran in H2O)</th>
<th>pH (LLB)</th>
<th>pH (FITC-Albumin in H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 % HA</td>
<td>7.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 % HA</td>
<td>6.82</td>
<td>6.40</td>
<td>7.06</td>
<td>7.44</td>
</tr>
<tr>
<td>1.0 % HA</td>
<td>6.80</td>
<td>6.45</td>
<td>6.95</td>
<td>7.44</td>
</tr>
<tr>
<td>1.5 % HA</td>
<td>6.73</td>
<td>6.45</td>
<td></td>
<td>7.43</td>
</tr>
</tbody>
</table>

The pH-meter was calibrated at the beginning of each measurement series.

4.1.2 Rheological data

In Figure 4.1, the storage and the loss modulus of three solutions of different concentrations of hyaluronan in PBS are presented resulting from frequency sweep tests at 25°C and 37°C.

Both the scales are logarithmic.
In Figure 4.1 can be noticed that both of the moduli increase with increasing concentrations of hyaluronan. At low deformation rates the viscous part of the response is dominating in the 0.5% HA sample, which is already reduced in the 1% and is not the case for the 1.5% sample. One can see that the influence of the temperature on the plateau of the storage and the loss moduli decreases with increasing concentrations. The storage modulus $G'$ becomes independent of the temperature at high frequency rates, but at lower frequencies both moduli decrease with increasing temperatures. The cross-over point between $G'$ and $G''$ is
moved to higher frequencies if the temperature increases and the concentration stayed constant. In the 0,5% HA sample, the storage and the loss modulus are of the same order of magnitude. The gap between them is already increased in the 1% sample, and is an order of magnitude different in the 1,5% sample. A strong entangled structure is formed if the concentration is higher than the critical overlap concentration $c^*$ (~0,5 mg/mL). If we heat up the samples, the material becomes softer. The results of the amplitude sweep test that we performed on the hyaluronan in $H_2O$ samples were similar to the values presented by [10] (the results are not stated in this report).

4.2 FRAP-experiments

Before the diffusion results are presented, the problems with the sample preparation of the latex beads are described.

4.2.1 Amine-modified Latex beads

It was not possible to do good FRAP-measurements in the samples with the ALB since it was noticed that the beads were not homogenous dissolved into the medium. Aggregates were lying on the bottom and were ranked in strings. Images were taken of these aggregates in the different concentrations as can be seen in Figure 4.2 and images with the CLSM are displayed in Figures 4.3, 4.4 and 4.5.

FIGURE 4.2: Amine-modified latex beads in 1% (left) and 1,5% (right) HA dissolved in $H_2O$.

FIGURE 4.3: Images of ALB in 0.5 % HA in $H_2O$ (scale = 30 µm).
Several attempts were done to let the aggregates disappear. A 1.3% HA sample was heated up to 65°C, but that did not let the aggregates diminish or disappear.

There was tried to avoid these aggregates by adding the latex beads to the hyaluronan solutions 16 hours before the examination with the microscope. A 1% HA in H₂O solution was prepared and stored on the mechanical shaker in the fridge. The solutions after adding the ALB. In the hyaluronic acid in PBS sample, the probes started to coagulate and form strings after 5 minutes while the beads in the H₂O sample stayed as a homogenous layer above the sample (Figure 4.6).
The next morning it was noticed that the probes had formed a layer above the H₂O sample and did not diffuse into the hyaluronan solution (Figure 4.7). The latex beads in the PBS sample were still in clots. While putting the thin layer above the sample into an aluminum cup for microscopic analysis, the film started to roll up. Images were taken with the CLSM (Figure 4.8).
Solutions of the ALB were prepared in PBS and in H$_2$O. These solutions looked cloudy as can be seen in Figure 4.9. Visible air bulbs/aggregates started to appear after one hour. These were investigated 2 days later in a secure seal between two cover glasses. Aggregates were found in both the PBS and the H$_2$O sample, but less frequently in PBS. CLSM-images of these aggregations can be seen in Figure 4.10 and 4.11.

**FIGURE 4.9: Solutions of 25 µL probes in 475 µL H2O or PBS (HLB = ALB).**

**FIGURE 4.10: ALB in H2O (scale$_{left}$ = 30µm, scale$_{middle}$ and right = 50µm)**

**FIGURE 4.11: ALB in PBS (scale$_{left and middle}$ = 30µm, scale$_{right}$ = 50 µm)**

We cannot impute the observed aggregations of the latex beads to one single reason. The hydrophobic latex beads probably did not ‘like’ the aqueous environment of the solvent and started to clot together. Since the aggregates of the amino-modified latex beads also
occurred in an aqueous environment without hyaluronan, the coagulations could not be caused exclusively by very strong interactions between the negatively charged groups of the hyaluronan strands and the positively charged probes. The clots were lying in a pattern. If a good look is given at the image in the right down corner of Figure 4.5, the possibility arises that the aggregates (bright zones) were too big to move in the hyaluronan network and stayed stuck in the caves between the hyaluronan chains (dark zones).

Future research should focus on the exact influence of hyaluronic acid in this process and the characteristics of the interaction of the latex beads with the hyaluronan chains. Different sample preparation methods should be tried to prevent the formation of the aggregates: for example a different solvent or the use of ultrasonic treatment.

4.2.2 Carboxylate modified latex beads

One 1% hyaluronic acid sample was prepared with smaller latex beads (latex beads carboxylate-modified polystyrene L5155, Sigma-Aldrich, St. Louis, USA). We only prepared one sample to see if aggregates were formed. When the sample was taken out of the fridge, it contained a lot of small air bulbs. The bulbs disappeared or drifted to the surface during the 4 hours placement at room temperature. There was a slight formation of aggregates, but in contrast to the amine-modified latex-beads there were also beads in solution and it was possible to find good places for FRAP-measurements.

Since FRAP-measurements were possible, a 0,5% and 1,5% HA sample were prepared and put on the shaker for 2 days.

The 0,5% sample seemed to be very inhomogeneous as can be seen in Figure 4.12 in contradiction to how it looked with the bare eye. It was very difficult to find good places for FRAP-measurements.
FIGURE 4.12: Images of CLB in H$_2$O in 0.5% HA. Scale in upper left corner is 40µm and 50µm in the others.

The 1.5% sample seemed microscopically to be more homogenous although at some places strings could be seen with the bare eye.

Samples were filled in the aluminum cups carefully to include as little aggregates as possible and the FRAP-measurements were done in the most homogenous regions in the sample.

Aggregates were found in the samples where the probes were diluted in pure H$_2$O or PBS (Figures 4.13 and 4.14).

FIGURE 4.13: CLB in H$_2$O (scale left and middle = 50 µm, scale right = 30 µm).
4.2.3 FRAP-results

The absolute values of the diffusion coefficients of the probes in the 3 different systems are presented in Figure 4.15. The error bars in all the following graphs depict the 95% confidence interval of the measurements. The uncertainty due to the limited amount of measurements was taken into account by mean of the student T-test.

In Figure 4.15 can be seen that the absolute values of the diffusion coefficients of the FITC-dextran molecules are slightly higher in PBS than in H$_2$O at the same temperature. This is due to ionic interactions between the ions in the solution and the charges of the fluorescent probes. The FITC-dextran molecules shrink in PBS: their hydrodynamic radius decreases and the diffusion coefficient increases.
The hydrodynamic radius was calculated out of the measured diffusion coefficients and the Stokes-Einstein equation (Eq 2.2). It was not possible to perform FRAP-experiments on the ALB samples due to the formation of self-aggregates. In the following graphs and tables, this fluorescent probes are omitted. Observations and comments on the ALB can be found in Section 4.2.1, on CLB in Section 4.2.2, FITC-albumin in Section 4.2.4 and on the FITC-dendrimers in Section 4.2.5. The calculated hydrodynamic radii are given in Table 4.2.

**TABLE 4.2: Calculation of the hydrodynamic radius with the Stokes-Einstein equation (Eq 2.2) out of the diffusion coefficient measured by FRAP at 25°C and analyzed in Matlab with 20 frames.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent</th>
<th>$D$ ($10^{-12}$ m$^2$/s)</th>
<th>95% CI</th>
<th>Radius (m)</th>
<th>95% CI</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-Fluorescein</td>
<td>H2O</td>
<td>338,8481 ± 45,748</td>
<td>7,238<em>10$^{-10}$ ± 1,130</em>10$^{-10}$</td>
<td>376</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>371,3674 ± 70,886</td>
<td>6,320<em>10$^{-10}$ ± 1,491</em>10$^{-10}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 kDa FITC-dextran</td>
<td>H2O</td>
<td>57,6481 ± 1,272</td>
<td>4,254<em>10$^{-9}$ ± 9,601</em>10$^{-11}$ ±10000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>64,4464 ± 5,861</td>
<td>3,642<em>10$^{-9}$ ± 3,644</em>10$^{-10}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 kDa FITC-dextran</td>
<td>H2O</td>
<td>18,3814 ± 0,758</td>
<td>1,334<em>10$^{-8}$ ± 5,738</em>10$^{-10}$ ±70000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>33,6213 ± 1,976</td>
<td>6,981<em>10$^{-9}$ ± 4,358</em>10$^{-10}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLB</td>
<td>H2O</td>
<td>20,9110 ± 3,207</td>
<td>1,173<em>10$^{-8}$ ± 2,125</em>10$^{-9}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>22,0701 ± 4,840</td>
<td>1,063<em>10$^{-8}$ ± 2,987</em>10$^{-9}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-albumin</td>
<td>H2O</td>
<td>57,5428 ± 4,942</td>
<td>4,262<em>10$^{-9}$ ± 4,005</em>10$^{-10}$ ±66000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>54,7347 ± 7,416</td>
<td>4,288<em>10$^{-9}$ ± 6,720</em>10$^{-10}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-dendrimer</td>
<td>PBS</td>
<td>119,9609 ± 20,331</td>
<td>1,956<em>10$^{-9}$ ± 3,992</em>10$^{-10}$ ±12000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The fluorescence recovery of the probes in different concentrations of hyaluronan in H$_2$O was recorded and the diffusion coefficients were calculated in Matlab. We normalized these values for the diffusion coefficient of the probes in pure water at the same temperature by calculating the relative diffusion coefficient. In Figure 4.16, those relative values of the diffusion coefficients in H$_2$O are depicted. The values of the diffusion coefficient of the probes in hyaluronan in PBS at 25°C divided by the diffusion coefficient of the probes in pure PBS at 25°C are displayed in Figure 4.17 and those values for PBS at 37°C in Figure 4.18.
FIGURE 4.16: The relative diffusion coefficients of the probes in H$_2$O at 25°C. (error bars = 95% CI)

FIGURE 4.17: The relative diffusion coefficients of the probes in PBS at 25°C. The diffusion coefficients calculated from the data of 20 frames are displayed. (error bars = 95% CI)
The absolute and the relative diffusion coefficients generally decreased with increasing concentrations of HA as can be seen in the Figures 4.16, 4.17 and 4.18. This can be explained by the formation of a denser network that obstructed the probe molecules and slowed down their diffusion. The relative diffusion coefficient of the fluorescein samples did not decrease between the 0.5% and the 1% HA sample comparing the 95% confidence intervals, but in PBS in 1.5% HA, the relative diffusion coefficients drops down. It can be concluded that the negatively charged fluorescein probes are too small to be affected in their diffusion by the difference in hyaluronan concentrations between 0.5% and 1% HA but that 1.5% HA already starts hindering the probe in its diffusion.

If the concentration of hyaluronic acid increases, the diffusion coefficients decrease. The smaller mesh sizes of the hyaluronan network and the increased steric hindrance are the dominating factors. The negatively charged probes interact only little with the hyaluronic acid chains. Large probes can be trapped in the hyaluronan gaps because the negative carboxylic acid groups prevent them from passing.

The absolute diffusion coefficients increase with increasing the temperature from 25°C to 37°C due to a faster thermal motion of the molecules. All the probes, besides the 70 kDa FITC-dextran, keep their ratio $D/D_0$ constant independent of the temperature. This suggests that the network structure did not get changed by this temperature difference. Why the
relative diffusion coefficient of the 70kDa FITC-dextran molecule decreases with an increase in temperature is not known and more research and remake of the samples should be done to exclude faults in the sample preparation or the measurements.

Further comparing water and PBS at 25°C (Figure 4.16 and 4.17) slight increase in the diffusion coefficient in PBS is observed which is due to the electrolyte induced destiffening of the hyaluronan chains. That means that a more flexible network with bigger ‘pores’ is formed.

For Na-fluorescein, the FITC-dextran molecules and the latex probes the free-diffusion script (without taking probe-polymer interactions into account) was sufficient to describe the diffusion. Sticky interactions were observed in the FITC-albumin and the FITC-dendrimer sample [5].

4.2.4 FITC-albumin

The solutions of FITC-albumin in PBS looked slightly darker than those in H₂O, they foamed after vortexing and they needed a higher bleaching intensity (100% instead of 30% of the maximum laser intensity) at the same temperature.

If the mean value of the diffusion coefficient drops down with an increasing amount of analyzed post-bleach frames (this relates to the observation time) in Matlab, there is a chance for binding interactions influencing the systems recovery (Figure 4.19).

![FIGURE 4.19: Diffusion coefficient in function of the amount of analyzed frames, measured in a 1% HA sample in PBS at 37°C.](image)

It was necessary to describe the recorded recovery with a model that takes binding into account [45]. In the 1% hyaluronan sample in PBS at 37°C, the $K_{on}$ was calculated to be 0.075 s⁻¹ and the $K_{off}$ was 1.25 s⁻¹. This means that the average time of free diffusion between two
“sticky interactions” was 13.3 s and that the average time that a molecule was bound was around 0.8 s with a diffusion coefficient of 47.5 µm²/s.

### 4.2.5 FITC-Labeled dendrimers

The FRAP-measurements with the positively charged dendrimers were characterized by a high bleaching during recovery as can be seen in Figure 4.20. The intensity of the laser beam during scanning was lowered down, but if this setting was changed too much, the fluorescent field wasn’t homogenous anymore.

![Graph showing diffusion coefficients](image)

**FIGURE 4.20: FRAP-experiment of FITC-labeled dendrimers in PBS at 25°C. Notice the bleaching during recovery**

The diffusion coefficients were dependent on the number of analyzed frames as can be seen in Figure 4.21.
FIGURE 4.21: Diffusion coefficient in function of the amount of analyzed frames, measured in a 0,5% HA sample in PBS at 25°C.

Only the two first FRAP-experiments could be considered as usable for fitting to a binding-model. The script told us that there were some sticky interactions. The on-rate was estimated to be $0.08 s^{-1}$ and the $K_{off}$ was approximately $1 s^{-1}$ in the 0,5% HA sample at 25°C, giving a diffusion coefficient of $120 \mu m^2/s$.

Although it seems that the positively charged dendrimers also interacted with the negatively charged hyaluronic acid chains, these values are derived from only 1 measurement and cannot be considered as accurate values. The on rates and the off rates of the dendrimers were in the same range as the values for FITC-albumin. Due to the short duration of this master thesis, there was no time left to investigate why such a high level of bleaching occurred during pre-bleaching and during recovery but it seems that the probes were very sensitive to bleaching. Different laser intensities of the scanner were tested, but if this setting was lowered too much, the fluorescent plane was not homogenous anymore. No characterization has been performed yet on the range in which the relationship between the concentration of the probes and the fluorescence intensity is linear. The probes were diluted as much as possible to still have a good fluorescent signal, so inner filtering can be excluded. No tests of the bound amount of FITC per mol of dendrimer have been performed. It could be a topic for further research to characterize the FITC-dendrimer probes and to study their possible electrostatic hindered diffusion in hyaluronan networks.

4.2.6 Critical remarks

It is hard to draw statistically correct conclusions out of samples which were prepared only once or twice. 5-10 FRAP-curves were recorded per sample and the student T-tables were used to calculate the 95% confidence interval to count in this uncertainty. It is a good option to prepare in the future more samples since small differences in the sample preparation can occur. There possibly was an influence of the mechanical shaker on the formation of the hyaluronan networks. A possible sign for this was the presence of different amounts of air bubbles in samples with the same concentration of hyaluronic acid. The speed of the shaker was set at the lowest possible shaking rate without stopping the movement but this setting was very sensitive and hard to reproduce. The way in which the samples were placed on the stirrer could also have had an influence on the result.
More probes with varying charges could be tested in the future. Especially an extra positive charged probe could give us a lot of information. It is difficult to find probes that meets all the requirements (fluorescence induced by the wavelength of the laser light, positively charged, fluorescent in certain pH-range,...). The study started with experiments of well-known probes to build up knowledge about the hyaluronan system and confidence in the FRAP-measurements. To investigate ionic interactions, it would have been better if we could have studied more positive probes in more detail. As the positively charged commercial available probe (amine-modified latex beads) was found to be too large for diffusion measurements in hyaluronic acid, we decided to label the smaller positively charged dendrimers with FITC in the lab and use them as ‘home-made’ probes.

Although researchers have been investigating on these topics for years, a lot of research still has to be done. The further development of the microscopes and the FRAP-technique will enable this progression.
5 CONCLUSION

7 different probes (Na-fluorescein, 10 and 70 kDa FITC-dextran, amine-modified latex beads, carboxylate modified latex beads, FITC-albumin and FITC-labeled dendrimers) were chosen to prepare hyaluronic acid samples with in H₂O and PBS. 3 different concentrations were investigated: 0,5% HA, 1% HA and 1,5% HA. FRAP-measurements were performed with a confocal microscope and the recorded data were analyzed in Matlab. The following conclusions could be drawn:

- The absolute values of the diffusion coefficient of the probes increased when the samples were heated up to 37°C due to the increased thermal motion of the molecules.
- The Na-fluorescein molecules were too small to undergo interactions with the hyaluronan, even in higher concentrations. Their movement was not limited significantly by steric hindrance, as they could diffuse freely through the pores in the hyaluronan network and their negative groups were not attracted by the negative carboxylic acid groups.
- The uncharged FITC-dextran molecules are slowed down by increasing the concentration of hyaluronic acid. The absolute and the relative diffusion coefficients of the 70 kDa molecule are smaller than those of the 10 kDa FITC-dextran probes since their higher molecular weight makes them more sensitive to steric hindrance. They have a higher diffusion coefficient in PBS than in water due to shrinking and so decreasing the hydrodynamic radius. No interactions with the hyaluronan were observed.
- The amine-modified latex beads started clotting together. Different ways of sample preparation and handling were tested, but no positive effect was found. It was not possible to perform FRAP-measurements. It seems that the hydrophobic particles were too big to diffuse through the hyaluronan network and stayed stuck in the polymer instead.
- The carboxylate-modified latex beads started clotting together in an aqueous environment, but it was possible to perform FRAP-measurements. These results should be interpreted with a lot of care.
• Interactions between the positive parts of the albumin and the negatively charged hyaluronic acid were observed.
• ‘Sticky interactions’ were observed between the positive FITC-labeled dendrimers and the negative carboxylic acid groups on the hyaluronan strain. There was often a significant amount of bleaching during recovery, which made most of the measurements hard to analyze. More measurements are needed to be done to confirm the interactions.

Future research should focus on the diffusion characteristics of other positively charged fluorescent probes, the sample-preparation methods of the latex beads, the determination of the range of linear relationship between fluorescence intensity and the FITC-dendrimer concentration and further characterization of the FITC-dendrimers. In the end, this will lead to a better knowledge of the diffusion characteristics in hyaluronan, which could help in the development of hyaluronan-based pharmaceutical dosage forms for continued release.
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“Transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers: Influence
