Hydrogels of Poly(vinyl alcohol) and Nanocellulose for Ophthalmic Applications

Synthesis, Characterization, Biocompatibility and Drug Delivery Studies

GOPI KRISHNA TUMMALA

Hydrogels are commonly used materials in ophthalmic care as contact lenses, bandage lenses, corneal implants, and cornea regeneration scaffolds. Hydrogels can be produced by physical, chemical, or radiation crosslinking of hydrophilic polymers. Poly(vinyl alcohol) (PVA) is a hydrophilic polymer that has been long known to the scientific community and is used in ophthalmic formulations.

In this thesis, PVA was reinforced with nanocellulose to obtain self-standing hydrogels. Cryogelation technique was used to obtain transparent hydrogels from PVA dissolved in DMSO-water mixed solvent. The properties of these hydrogels were analyzed to explore the possibility of their application for ophthalmic use as a drug delivery vehicle and as cornea regeneration implant.

The results indicate that oxidized nanocellulose can be combined with PVA to produce transparent, elastic, macroporous and high-water content hydrogel lenses. The water-filled macroporous structure of these hydrogels aids with oxygen transport and can enhance comfort while worn. The developed hydrogel also features moderate UV-light blocking properties in addition to high transparency. Furthermore, it was observed that the light scattering due to surface roughness of the hydrogel increases with measurement time, due to the rapid evaporation of the water layer from the surface of the hydrogel film.

Mechanical analysis results revealed that the hydrogels exhibited a strain-induced stiffening behavior, which is usually noticed in hyper-elastic materials and collagenous soft tissues. The results of this study suggest that in order to mimic collagenous behavior, the material should possess high water content and a specific structural architecture combining soft and rigid elements as building blocks.

Furthermore, PVA-CNC composite hydrogel showed no toxic effects on the corneal cells in both direct and indirect contact studies. It was found that the hydrogel promoted cell attachment and was stable when sutured ex vivo to a porcine excised cornea.

To study enzyme-triggered drug release, hydrogel lenses loaded with chitosan-poly(acrylic acid) nanoparticles were exposed to lysozyme, an enzyme present in the eye. Nanoparticles were shown to disintegrate due to the hydrolysis of chitosan chains by lysozyme. Overall, with these distinctive properties, PVA-CNC hydrogel has great potential as an ophthalmic biomaterial for therapeutic and cornea regeneration applications.

Keywords: nanocellulose, poly(vinyl alcohol), hydrogel, cornea regeneration, therapeutic lens

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ISSN 1651-6214
ISBN 978-91-513-0285-0
urn:nbn:se:uu:diva-346478 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-346478)
Dedicated to The Lineage of Gurus;
Especially to Sri P J Thomas for instilling scientific temper in me
and to Prof. Tapani Vuorinen for giving new life to my dreams
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


V  **Tummala, G. K.;** Bachi, I.; Mihranyan, A. Role of solvent on structure, viscoelasticity and mechanical compressibility in nanocellulose-reinforced poly (vinyl alcohol) hydrogels. *In manuscript.*

VI **Tummala, G. K.;** Lopes, V. R.; Mihranyan, A; Ferraz, N. Biocompatibility of nanocellulose-reinforced PVA hydrogel with human corneal epithelial cells for ophthalmic applications. *In manuscript*


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My contributions to the included papers

**Paper I:** I participated in the planning of the study and performed the majority of the experimental work. I wrote the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper II:** I participated in the planning of the study and performed the majority of the experimental work. I wrote the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper III:** I participated in the planning of the study and performed material preparation, optical microscopy and fluorescent microscopy parts of the experimental work. I participated in the writing of the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper IV:** I participated in the planning of the study and performed all of the experimental work. I wrote the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper V:** I participated in the planning of the study and performed DSC and optical microscopy parts of the experimental work. I wrote the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper VI:** I participated in the planning of the study and performed majority of the experimental work. I wrote the initial draft, contributed to the analysis of data, and was part of the continued writing process.

**Paper VII:** I participated in the planning of the study and performed nanoparticle integration process, SEM imaging and fluorescent microscopy parts of the experimental work. I participated in the writing of initial draft, contributed to the analysis of data, and was part of the continued writing process.
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>ARS</td>
<td>Angle resolved scattering</td>
</tr>
<tr>
<td>BTDF</td>
<td>Bidirectional transmittance distribution function</td>
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<tr>
<td>CMOS</td>
<td>Complementary metal-oxide semiconductor</td>
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<tr>
<td>CNC</td>
<td>Cellulose nanocrystals</td>
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<tr>
<td>CNF</td>
<td>Cellulose nanofibers/fibrils</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DTAF</td>
<td>5-(4,6-dichlorotriazinyl) aminofluorescein</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate isomer I</td>
</tr>
<tr>
<td>HCE-2</td>
<td>Human corneal epithelial cells</td>
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<tr>
<td>MCC</td>
<td>Microcrystalline cellulose</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles of Chitosan-poly(acrylic acid)</td>
</tr>
<tr>
<td>PSD</td>
<td>Power spectral density</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl) alcohol</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plate</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethylpiperidine-1oxyl radical</td>
</tr>
<tr>
<td>TS</td>
<td>Total scattering</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<tr>
<td>WLI</td>
<td>White light interferometry</td>
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1. Introduction

The cornea is the transparent tissue that forms the surface of our eyes. Light refracts through the corneal surface, which gets focused onto the retina with the help of a self-adjusting lens present posterior to the cornea. The ability to see properly depends on the optical clarity of this transparent tissue. Tear fluid plays an important role in ensuring clear vision and healthy environment in the eye by maintaining a smooth refractive surface, by transporting nutrients and oxygen to the avascular cornea. Tear fluid not only cleans the conjunctiva and eye from time to time, but also contains components of the immune system like immunoglobulins, lactoferrin, lysozyme, polymorphonuclear leukocytes, etc.1-3

Any damage to corneal tissue due to accident or infection leads to impaired vision and may ultimately lead to blindness if not corrected. The World Health Organization estimates that 50 million people are blind worldwide and corneal damage and disease is the major cause of blindness after cataracts.4 In some areas of Africa as much as 90% of all blindness is a direct result of corneal pathology.5

The only major curative treatment available for corneal blindness is corneal transplant surgery. Although the risk of rejection is low due to the non-vascular nature of the cornea,6 the biggest problem is the shortage of donor corneas and the logistics associated with it. Furthermore, the complications associated with donor-derived infections are a major concern.7-8 The other alternatives that are being explored are tissue engineered corneas,9 synthetic corneas,10-11 and hydrogels that serve as a matrix for host tissue regeneration.12-14 The best, cost-effective, devoid of transferable infections, and easily distributable solution is a biomimetic implant that can prompt the host tissue to regenerate on its own.

Apart from corneal blindness, ophthalmic medical management is the other major ophthalmic challenge that the public healthcare system is facing. Eye drops in the form of suspensions and solutions are the major drug delivery options for ophthalmic medical management. The low bioavailability of the drug due to short residence time is a major drawback. At present, topical drug formulations and intravitreal injections are common treatment strategies. At times, ophthalmic inserts in the conjunctival sac and implants are used to achieve improved drug delivery.15 These pathways are invasive and are associated with complications. Ideally, a drug-eluting soft contact lens
with controlled drug release could help to mitigate therapeutic management challenges that arise due to low bioavailability of drugs.

Various synthetic and natural polymer-based hydrogels are used in ophthalmic research. Polyvinyl alcohol (PVA) is one such synthetic polymer that has been long known to the scientific community and is used in biomedical applications and ophthalmic pharmaceutical formulations. PVA-based hydrogel has very good potential to be a therapeutic contact lens and could become an alternative to corneal transplant surgery. In these applications the hydrogel should be able to withstand mechanical forces during handling and suturing. Given the softness of the hydrogel, this is a challenging demand from the materials perspective.

Hydrogels are inherently weak because of their high water content, and often require cross-linking or reinforcement. Although several suitable materials can be used as reinforcing agents to enhance mechanical properties, the choice should also be based on the optical properties and biocompatibility of the final composite material. In this context, nanocellulose, which was previously shown to be biocompatible, was chosen as the reinforcing material. The availability of a high surface area and abundant hydroxyl groups makes nanocellulose a good choice from a material interaction standpoint with PVA.

In this thesis, PVA hydrogels reinforced with nanocellulose were investigated from the perspective of being used as a therapeutic lens as well as an implant for cornea regeneration. Their properties pertain to several practical aspects from an application viewpoint, which are presented in this work. Specific aims of this thesis are listed in the following section.
2. Aims of the thesis

The work presented in this thesis focuses on the development of polyvinyl alcohol (PVA) and nanocellulose-based composite hydrogels as well as the evaluation of their properties, in order to examine their plausible use in ophthalmic applications. The specific aims of the included papers are as follows.

- To develop a reproducible synthesis procedure for a PVA-CNC composite hydrogel and broadly investigate the key properties of the latter to determine the possibility of its application as an ophthalmic biomaterial (Paper I).
- To study the effect of solvent composition and nanocelluloses of different aspect ratios on the physicochemical properties of the hydrogel, with special focus on optical profile (Paper II).
- To analyze the light scattering phenomenon in PVA and nanocellulose composite hydrogels and evaluate the influence of the layer of surface water on it (Paper III).
- To enhance the mechanical properties of the PVA hydrogel by introducing nanocelluloses and develop a model describing the strain-induced stiffening behavior of the composite hydrogels (Paper IV).
- To study the effect of solvent composition and CNC reinforcement on the structure, viscoelastic properties, and mechanical compression properties of the composite hydrogel (Paper V).
- To evaluate the corneal epithelial cell response to the PVA-CNC composite hydrogel and validate the non-toxic and cytocompatible profile of the hydrogel (Paper VI).
- To develop nanoparticle-loaded hydrogel-based contact lenses that could be used for ophthalmic drug delivery (Paper VII).
3. Ophthalmic Inserts

3.1 Contact lenses

Vision corrective lenses include prescription eyeglasses and contact lenses. Compared to eyeglasses, contact lenses are lightweight and virtually invisible. Contact lenses remain an important part of modern eye care and culture. More than 85 million people worldwide wear contact lenses for corrective, cosmetic, and therapeutic purposes.23

Contact lenses can be broadly classified into two types depending on the manufacturing material: hard and soft contact lenses. Hard contact lenses are generally made of polymethyl methacrylate (PMMA). These are first generation contact lenses developed in the 1940s.24 These have low oxygen permeability, which may lead to unwanted clinical events such as hypoxia of the cornea and various types of edema. Because of this reason, they cannot be worn for a long duration. The quest for oxygen permeability led to the development of oxygen permeable contact lenses by using different polymers or techniques.24

The breakthrough in the development of soft contact lenses was achieved when 2–hydroxyethyl methacrylate cross-linked with ethylene glycol dimethacrylate and water resulted in a hydrogel.25 They were flexible, and wearers felt comfortable wearing them after a very short period of time, unlike rigid contact lenses, which need some time for adaptation. The advantages of soft contact lenses are increased oxygen permeability, lens wettablility, and an overall improvement in comfort. FDA classifies soft contact lenses in four major groups, depending on their water content (high vs. low) and type of polymer (non-ionic vs. ionic) as shown in Figure 1.

In 1980, silicone-based hydrogels were developed for soft contact lenses.24 The advantages were greater comfort and increased oxygen permeability, and these did not depend on the water content of the lens. Disadvantages include marginal stiffness and hydrophobicity. Hydrophobic materials have disadvantages such as a lack of comfort due to lower wettablility, adsorption of lipids and surface damage due to high electrostatic charging.26 Hydrophobicity was overcome either by surface modification or incorporation of internal rewetting agents. At present, silicone hydrogels are most common and have excellent oxygen permeable properties superior to other materials,24 but the comfort while wearing these lenses is inferior owing to its limitation on water content. It has been argued that an equilibrium in wa-
Water content

High (>50%)           Group II           Group IV

Low (<50%)            Group I            Group III

Non-ionic            ionic

Polymers type

Figure 1. Classification of soft contact lens materials based on FDA guidelines.

3.2 Therapeutic lenses

Eye drops in the form of suspensions and solutions are the major drug delivery-options for ophthalmic medical management. The low bioavailability of the drug due to short residence time is a major drawback. Ideally, a drug-eluting contact lens could help to mitigate therapeutic management challenges that arise due to low bioavailability. The idea of using contact lenses for ophthalmic drug delivery has been around for more than 40 years.

A majority of these lenses employ a soaking technique to enrich the lens with a drug, which is later released onto the eye. The major disadvantage with this strategy, however, was the initial burst release that was observed during the first hour when the lens was applied, in which the majority of the drug was released. This system essentially supplies the drug for only a couple of hours. Several strategies were employed to enhance the drug release time such as molecular imprinting, addition of diffusion barriers and embedding nanoparticles loaded with drugs into the lens. Although controlled release with nanoparticle formulation is a better strategy, the drawback is that the drugs may diffuse from the particles and lens prematurely during storage.
3.3 Cornea regeneration materials

The cornea is the transparent tissue that forms the surface of eyes. Its main function is to focus and transmit light. Injury, disease, or hereditary conditions can cause clouding, distortion, or scarring of the cornea, which may all interfere with vision. To restore vision and maintain structural integrity of the eye, an implant or a corneal transplant is necessary.

Hydrogels were previously used for corneal implantation and polyhydroxy ethylmethacrylate (PHEMA) is the prominent material used. Chirila and co-workers created the first “core and skirt” hydrogel, based on keratoprosthesis using PHEMA, and is a device known commercially as Alpha-Cor. Despite being classified as a hydrophilic polymer, PHEMA is still relatively hydrophobic compared to other hydrogels such as PVA, which can attain an equilibrium water content of over 80%. Hydrophobicity makes materials more susceptible to nonspecific protein adsorption, which has been proposed to be the molecular event that triggers calcification. Many groups have studied collagenous hydrogels, as corneal stroma is predominantly composed of collagen. Cornea regeneration biomaterials, including recombinant human collagen-based, fibrin-based, silk-based, and self-assembled corneal implants, have been previously described in the literature.

The thickest layer of the cornea, which is the stroma, is primarily made of collagen and proteoglycans. This is the layer that will be replaced with the bioengineered material envisioned for implantation. Owing to the structural similarities to extracellular matrix and their three dimensional framework for cell proliferation and survival, hydrogels have unique desirable properties.
4. Hydrogels

Hydrogels are a porous water swollen network formed from hydrophilic monomers either by physical, chemical or radiation cross-linking, which when formed does not dissolve in water. Physical crosslinking includes freeze thawing induced crystallization, van der Waals interactions, ionic interactions and hydrogen bonding between the polymer chains. Chemical crosslinking agents like formaldehyde, glyoxal, glutaraldehyde, maleic acid, borax etc. are used for covalent crosslinking. Radiation crosslinking typically includes gamma radiation, electron beam radiation and UV radiation. Several synthetic and naturally derived polymers are used to develop implantable hydrogels, such as polyacrylic acid derivatives, polyethylene oxide, polyphosphazene, polypeptides, collagen, gelatin, hyaluronate, alginate, chitosan and fibrin.

Hydrogels are commonly used in eye-care as lenses to correct refractive errors, as cosmetic and decorative ophthalmic devices, as therapeutic drug delivery vehicles, bandage lenses, scleral buckling materials, cornea regeneration scaffolds, and even integrated ophthalmic wireless electronic sensors for diagnostic purposes.

The choice of hydrogel material for an ophthalmic application depends on several factors such as optical, mechanical strength, biocompatibility, biodegradability, surface properties, and permeability. Poly(vinyl alcohol) is one such material that has proven itself as a flexible, biocompatible matrix that is capable of addressing many demanding medical needs.

4.1 Polyvinyl alcohol

Poly(vinyl alcohol), PVA, is synthesized by partial hydrolysis of poly(vinyl acetate) produced by radical polymerization of vinyl acetate monomers. It is a hydrophilic polymer rich in pendant hydroxyl groups that act as attachment sites for biomolecules. It can be cross-linked physically or chemically to produce a three-dimensional polymer network. Typically, radiation cross-linking or photo-curing was used to produce PVA hydrogels as an alternative to chemical cross-linking. Chemical cross-linking is less preferable, due to the toxic nature of the cross-linking agents like formaldehyde, glutaraldehyde, glyoxal etc. used in the process.
PVA hydrogel is elastic, stronger than most other synthetic gels, has a low coefficient of friction, and has structural properties similar to collagen. Its availability and unique chemical and physical properties have led to its inclusion in many implants and biomaterials that have been used in the fields of keratoprosthesis, vascular prosthesis, articular cartilage and meniscus regeneration among others.

PVA has long been used in ophthalmic compositions. PVA films and gels have been reported as ophthalmic inserts in the lower conjunctival sac when loaded with antibiotics. Owing to its great alterability, PVA sponge is used as a surgical spear in micro-surgical procedures, such as eye surgery.

4.1.1 PVA as soft contact lens material

Although contemporary, commercially available thin soft contact lenses have good oxygen permeability and a good level of comfort, one of the issues faced is the development of proteinaceous deposits on the polymer matrix. Increasing the water content to reduce protein deposition has so far not been feasible, as hydrogels are in general mechanically weak and would further compromise their mechanical properties during use and handling. Hence, the benefit of high water content needs to be balanced by sufficient mechanical strength and flexibility. In this context, transparent PVA hydrogels are interesting candidates for affordable, biocompatible, and mechanically strong contact lens materials with a high water content. This will be the subject of the present work.

The idea to use PVA hydrogels for contact lenses and keratoprosthetic applications was first explored by Japanese researchers from Kyoto University in the late 1980s-early 1990s. Hyon et al. developed a method to make a transparent PVA hydrogel using solvent mixtures. Kita et al. evaluated the PVA hydrogel material for soft contact lens application and concluded that in addition to good mechanical strength and oxygen permeability, it also had low protein absorption, high water content, and transparency. In 1998, Müller from Novartis patented a technology to develop PVA-based hydrogels for soft contact lenses (Nelfilcon A), involving the chemical modification of PVA, i.e. poly(vinyl alcohol) partially acetalized with N-((formyl methyl)acrylamide) to enable cross-linking by UV irradiation in molds.

4.1.2 PVA-based ophthalmic implants

In 1997, Tsuk et al. studied the application of PVA in keratoprosthesis in vivo. Tamura and co-authors showed that PVA is biocompatible and hemocompatible and therefore has great potential in regenerative medicine. In 2001, Wu et al. developed a synthetic cornea with a transparent PVA-based hydrogel optic center and has demonstrated that limbal epithelial cells can
migrate onto the synthetic cornea in a rabbit model. In 2014, He et al. developed a PVA/chitin-based composite hydrogel, which showed good in vitro biocompatibility, flexibility, and transparency.

PVA-based hydrogels have been previously tested in cartilage and orthopedic applications by several research groups. On the other hand, little work has been done to develop PVA hydrogel-based implants for ophthalmic treatments. Therefore, owing to PVA hydrogel’s biocompatibility, transparency, strength, and oxygen permeability, it is a good idea to explore plausible applications of PVA hydrogel as an implant material in treating eye ailments.

4.1.3 Cryo-gelation of PVA

Previously, it was known that DMSO is a better solvent for PVA than water, and mixtures of DMSO and water show a sharp decrease in freezing point. PVA chains are more coiled in an aqueous solution, whereas they are extended in a DMSO solution. Upon cooling of PVA in a mixed solvent, the solubility of PVA is reduced, resulting in gradual phase separation and eventually local cross-linking of formed PVA crystallites. Once an infinite network is formed, it is difficult for the solvents to undergo phase separation, resulting in a three-dimensional gel structure. There are previous reports suggesting that the overall crystallinity is the lowest for a DMSO:water composition of 70:30. This is because there are clusters of 1DMSO.2H2O that exist in the mixed solvent system and reduce the interaction of the solvent with PVA.

Factors such as PVA concentration, degree of deacetylation, molecular weight, solution composition, and quenching temperature are important for gelation. The strength of the PVA hydrogel primarily depends on its molecular weight and concentration. Strong hydrogels can be obtained from high molecular weight PVA but are difficult to manufacture, expensive to purchase, and difficult to process due to their high viscosity. In order to solve this problem, low molecular weight PVA hydrogels can be reinforced with compatible additives to enhance its strength. In this work, TEMPO oxidized nanocellulose is used to reinforce the PVA hydrogels.

4.2 Nanocellulose

Cellulose is the most abundant natural polymer on earth, that has been used for human needs for thousands of years. Apart from trees and plants, cellulose can be obtained from several other sources such as tunicates, bacteria, and algae. The elementary units of the cellulose consist of highly ordered crystalline regions and amorphous regions that are less ordered in structure. The hierarchical structure of wood can be seen in Figure 2; it is composed of
cellulose chains, which are arranged in an orderly fashion, similar to a synthetic composite material, with lignin as the gluing material. Nanocellulose is extracted from cellulose pulp via physical or chemical methods or a combination of both methods. Nanocellulose is highly sought as a reinforcing material, because of its high mechanical strength, availability of reactive hydroxyl groups, biocompatibility, and high specific surface area.

Figure 2. Illustration showing the hierarchical structure of wood. (This figure is adapted from Robert J. Moon’s work)

Nanocellulose can be broadly classified into two types: (1) cellulose nanocrystals (CNCs, also called whiskers) – which are short rigid rod-like structures with high crystallinity and (2) cellulose nanofibrils (CNFs) – which are longer, flexible, and have a higher aspect ratio (Figure 3). CNCs are produced by acid hydrolysis of cellulose where amorphous regions are dissolved, leaving out highly ordered crystalline regions. CNCs can be oxidized to introduce carboxyl groups over them. The suspension of oxidized CNCs is a viscous free flowing gel around 0.5 wt% (Figure 3c). CNFs are typically produced by a two-step process where a mild pretreatment step (chemical or enzymatic) loosens up the cellulose network followed by mechanical isolation of fibrils, by high pressure homogenization or grinding. CNF gel is self-standing around 0.5 wt% due to entanglement of high aspect ratio nanofibrils (Figure 3d). In this text, both carboxylated CNCs and CNFs, henceforth will be referred to as CNCs and CNFs for simplicity.

Chemical pretreatment induces addition of charges onto the cellulose chains, which facilitates disintegration by disrupting hydrogen bonds and creating repulsion among the cellulose chains. TEMPO oxidation is one of the common methods currently used as a pretreatment step. This is where carboxyl groups are created via an aldehyde intermediate by selectively oxidizing the primary alcohol present at C6 position of the glucose unit in a cellulose chain.
Figure 3. AFM images and physical appearance of TEMPO oxidized (a), (c) CNC at 0.58 wt%, and (b), (d) CNF at 0.53 wt% (reprinted from Paper IV).

TEMPO is a water soluble commercially available stable nitroxyl radical that acts as a catalyst in the oxidation reaction. Though it is a weak oxidant, the intermediate oxoammonium cation generated during the reaction has higher oxidation capacity. In TEMPO mediated oxidation, additional oxidants such as NaClO, NaClO₂ or NaBrO are used to generate the oxoammonium cation from reduced TEMPO continuously. Two typical TEMPO oxidation systems are in practice⁹⁸; one with NaClO/NaClO₂ in water at pH 4.8-6.8⁹⁹ and the other with NaBr/NaClO in water at pH 10-11¹⁰⁰. Modified TEMPO, like 4-acetamido-TEMPO can also be used for oxidation of cellulose¹⁰¹ to obtain longer CNFs than with TEMPO oxidation.

4.2.1 Nanocellulose as mechanical reinforcement

The scope of hydrogel applications is often severely hindered by their poor mechanical behavior. To enhance the mechanical properties of hydrogels, reinforcing materials such as carbon nanotubes,¹⁰² nanocellulose,¹⁰³ clay,¹⁰⁴ and metallic particles¹⁰⁵ have been used. The high surface area of the rein-
forcing materials enables them to have increased interaction with the polymer matrix, resulting in enhancement of mechanical properties.

Although various materials could potentially be used for reinforcement, the greatest challenge is to ascertain that the optical properties of the final product meet the requirements for ophthalmic use. Previous studies have shown that native cellulose is an excellent reinforcing agent for the PVA polymer matrix.\textsuperscript{106-109} Typically, incorporating native cellulose into PVA hydrogels would reduce the transparency of the composite and could result in a patchy-looking material. On the other hand, cellulose may be rendered transparent in the form of nanocellulose, which when properly blended with PVA could result in a suitable product. In particular, nanocellulose produced from TEMPO-oxidized cellulose, is transparent and has a high water-holding capacity. Furthermore, TEMPO-oxidized cellulose was shown to be cytocompatible in recent studies.\textsuperscript{20-22}

The unique properties of PVA and nanocellulose make the composite hydrogel a good contender for ophthalmic applications.
5. Experimental

5.1 Chemicals

Microcrystalline cellulose - Avicel PH 101 (MCC), 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), NaBr, NaCl, NaClO, NaOH, poly vinyl alcohol (Average Mw 146,000-186,000 and degree of saponification 99.9%) (PVA), dimethyl sulfoxide (DMSO), aqueous hydroxyl amine (50%), chitosan low molecular weight (Mw: 50-190 kDa, degree of deacetylation 75-85%), anhydrous acrylic acid, fluorescein isothiocyanate isomer I (FITC), sodium dodecyl sulfate, ethanol, methanol, HCl, CH₃COOH, isopropanol, buffer solution pH 7.00 (potassium hydrogen phosphate/sodium hydroxide), acetic acid, Dulbecco’s phosphate buffered saline (PBS), lysozyme, gamma-globulin, bovine serum albumin, live-dead assay kit were all purchased from Sigma Aldrich. Potassium peroxydisulfate (97%, K₂S₂O₈) was purchased from Alfa Aesar, GE. Micro BCA protein assay kit was purchased from Thermo Scientific. Alamar-blue (AB) cell viability reagent was purchased from Invitrogen. Keratinocyte serum free medium and supplements were purchased from ThermoFisher Scientific. All the chemicals used were of reagent or analytical grade. Deionized water was used in all the experiments.

5.2 Materials preparation

**TEMPO mediated oxidation of cellulose:** Pharmaceutical grade MCC (Avicel PH 101, FMC Corp.), obtained by acid hydrolysis of native cellulose, was oxidized according to a previously verified method to produce carboxylated CNCs. In short, 5 g of MCC was dispersed in 400 mL of deionized water and 90 mg of TEMPO. To this, 1 g of NaBr was added, which was stirred into 100 mL of deionized water. The mixture was stirred at 500 rpm using a magnetic stirrer. To this mixture, 10 mL of 10% wt NaClO was added at intervals of 30 min for 3.5 hours. The pH of NaClO was adjusted to 11 prior to addition using 1 M HCl. Further, 1 M NaOH was used to maintain the system pH at 10.5 for the entire period of the reaction. The reaction was quenched after 3.5 hours by adding 10 mL of ethanol. Cellulose was washed 3 times with deionized water by centrifugation and then dialyzed for 3 days. Cellulose was then collected by centrifuging and was dispersed in
deionized water by ultrasonication (Vibracell 700 W, 20 KHz, USA) for 5 min to obtain a transparent gel. The CNC solids content was 0.58 ± 0.198% wt.

Cellulose nanofibrils, CNF, were prepared from never-dried spruce sulfite pulp (provided by Nordic Pulp in Säffle, Sweden) using 4-acetamido-TEMPO as a catalyst based on the method described by Tanaka et al.101 The pulp (10 g) was suspended in 0.1 M acetate buffer (1000 mL, pH 4.8), which was stirred using an overhead stirrer at 150 rpm until fully dispersed. Thereafter, 4-acetamido-TEMPO (1 mmol) and sodium chlorite (0.1 mol) were added until dissolution. A buffered (pH 4.5) 30 mL of a 2 M NaClO solution (10.0 mmol) was added to the reaction flask in three steps, 10 mL each/2 hours. The reaction flask was maintained under nitrogen atmosphere over a period of 48 hours at 60 °C with continuous stirring. The oxidation was followed by washing thoroughly with acetate buffer and distilled water under vacuum filtration conditions. Afterwards, the pulp fibers were mechanically dispersed in deionized water at 1% wt and passed through a microfluidizer M-110EH (Microfluidics Ind., USA) for a total of four passes. The first two were through a set of “large pore” chambers (400 µm followed by 200 µm). The second set of passes were through “small pore” chambers (200 µm followed by 100 µm) with dilution in between. The obtained nanofibril suspension had a solid content of 0.53 ± 0.023% wt.

Preparation of hydrogel: To produce the hydrogels, 3 g of PVA was added to a mixed solvent system of DMSO and deionized water so that a concentration of 10% wt was obtained. The composition of solvent was varied by changing the mass ratios of DMSO to water so that the following mixtures were obtained: 80:20, 70:30 and 60:40. PVA solutions were obtained by heating the mixture at 100 °C under stirring in an oil bath for about 2 hours. For the preparation of a composite hydrogel, a nanocellulose gel equivalent to 30 mg dry weight was added to the PVA solution and further mixed for an hour at 100 °C. The homogenous and transparent solutions obtained by this method were cast into respective polypropylene molds and allowed to gel at -20 °C for 24 hours. The formed gels were collected from the molds, and the DMSO was exchanged with deionized water by dialyzing the gels in excess water for at least 48 hours. The obtained products after washing were stored in deionized water for further testing.

Chitosan nanoparticles synthesis: The nanoparticles were synthesized by template radical polymerization according to Hu et al.110 as follows; 3.00 mmol of chitosan was added to 50 mL of acrylic acid solution, containing an equimolar amount of acrylic acid, during magnetic stirring in a 250-mL three-necked round bottom flask. The chitosan was allowed to dissolve under nitrogen stream at room temperature until a clear solution was obtained, at which point 1.00 mmol K2S2O8 was added. The solution was allowed to
polymerize for 2 h at 70 °C until the suspension turned opalescent. The pH was kept at around 4 during the whole polymerization procedure by addition of 0.1 M HCl or 0.2 M NaOH. The finished nanoparticle suspension was then finally filtered and dialyzed using a dialysis cellulose membrane, with a molecular cut-off of 14 kDa for 24 h in 2000 mL of deionized water with continuous magnetic stirring (150 rpm).

**Fluorescent labeling of chitosan nanoparticles:** FITC-labeled chitosan was prepared according to a procedure employed by Huang et al. by dissolving 1.0 g of chitosan in 100 mL 0.1 M acetic acid in a 500-ml round-bottomed flask during continuous magnetic stirring. A total of 100 mL of methanol was then slowly added followed by dropwise addition of 50 mL of FITC dissolved in methanol. The solution was left to mix in the dark for 3 hours at which point the polymer was precipitated by addition of approximately 50 mL of 0.2 M NaOH. The obtained precipitate was then washed with a methanol solution (70:30 v/v% methanol to deionized water) and pelletized by centrifugation at 4000 rpm for 10 minutes. The washing and pelletization procedure was repeated until a clear and uncolored supernatant was obtained. The chitosan pellets were then redissolved in 100 mL of 0.1 M acetic acid by magnetic stirring and the acquired yellow solution was dialyzed (molecular cut-off of 14 kDa) in 2000 mL of deionized water for three days in the dark. The dialyzed chitosan solution was then finally freeze-dried and stored in the dark for further use. FITC-labeled nanoparticles were then synthesized from this chitosan.

**Integration of FITC labeled chitosan nanoparticles into hydrogels:** A total of 3.0 g of PVA was dissolved in 21.6 g of DMSO by gradual heating to 100 °C in an oil bath with continuous stirring. A total of 2.29 g of CNC-suspension, which was obtained from TEMPO oxidation of MCC, was then diluted with 3.14 g of FITC-labeled nanoparticle suspension and homogenized before being added to the clear PVA-solution. The mixture was left for an additional hour at 100°C in the dark while stirring. The obtained clear yellow tinged solution was then heated to 120 °C for 10 min and degassed by vacuum before being cast in polypropylene molds and left to gel at -20 °C for 24 h. The hydrogel lenses were then dialyzed for 48 h in the dark with 1000 mL of 10 mM saline solution and then stored in deionized water.

The formulation of labeled nanoparticle loaded, pure PVA lenses were made according to the above-mentioned procedure but with a small difference; 5.4 g of FITC-labeled nanoparticle suspension was directly added to 3.0 g of PVA and a 21.6 g DMSO solution at the beginning of the synthesis.
5.3 Characterization

5.3.1. Structural characterization

**Atomic force microscopy of nanocellulose:** The atomic force microscopy images were acquired in air using a Dimension Icon (Bruker, Germany) instrument. The samples were mounted on mica slides, by first pre-coating the surface with 0.1% wt poly(L-lysine) solution and then fixing a dilute (0.2% wt) dispersion of nanocellulose onto the mica surface by gentle drying. The images were acquired in the peak-force tapping mode, using the manufacturer’s ScanAsyst Air cantilever and ScanAsyst automatic optimization algorithm.

**Differential scanning calorimetry:** In order to observe the thermal events during gelation, polymer solutions were characterized by differential scanning calorimetry, after equilibrating them at room temperature for about 20 minutes. A Mettler DSC 3 Star® system (Mettler Toledo, Greifensee, Switzerland) equipped with a cooling accessory (Huber-TC 100, Germany) was used. Samples of 0.1 to 1.5 mg were weighed using an analytical balance (Mettler Toledo-XS105, Switzerland) and sealed in 40-µL aluminum pans with lids. An empty pan was used as a reference. Heat flow measurements were made at a rate of 10 Kmin⁻¹. The samples were subjected to freezing between 20 °C and -70 °C. All experiments were performed under nitrogen gas at a flow rate of 50 mL min⁻¹.

**Scanning electron microscopy:** The Carl Zeiss Merlin FEG-SEM instrument was used for electron microscopy. The samples were frozen in liquid nitrogen and then carefully freeze-dried to avoid shrinkage prior to analysis. The samples were mounted on aluminum stubs using adhesive carbon tape and sputtered with a thin layer of Au/Pd to minimize charging during imaging.

**Optical microscopy:** An Olympus AX70 microscope was used to acquire images of the hydrogels. For microscopy 12.5-mm-thick disc-shaped samples were used. The microscope was mounted with a CCD camera, and the DeltaPix software program was used to acquire the images. IF 550 green contrast color filter was used for better visualization.

**Atomic force microscopy of hydrogels:** A Bruker BioScope Catalyst instrument with a Bruker PFQNM-LC-A-CAL probe was used to obtain the images in a field of 50×50 µm². The probe is 17 µm tall, has a 65-nm tip radius, and a spring constant of 0.1 N/m, which makes it suitable for measuring soft materials. All the samples were mounted in a petri dish, and then submerged in Milli-Q water prior to and during the measurement. The imag-
es were acquired in peak-force tapping mode. Post processing of the images and surface roughness calculations were carried out in Bruker’s NanoScope Analysis v 1.6 software.

**Fluorescence microscopy:** CNC and CNF were labelled with DTAF, according to a previously reported procedure.\(^{112}\) Both CNC (500 mg) and CNF (500 mg) were added to DTAF (7.5 mg) under alkaline conditions (0.2 M NaOH) for 24 h, in the dark with magnetic stirring. The mass ratio of both CNC and CNF to DTAF were kept constant (500 mg CNC/CNF: 7.5 mg DTAF). The volume of the reactions depends on the initial concentration of the nanocellulose suspension. Solid NaOH and DTAF were added to the CNC/CNF suspension. The volume of the reaction was adjusted appropriately to obtain a 1 wt % suspension of CNC/CNF. To remove the unreacted DTAF and NaOH, samples were washed with deionized water by centrifuging 6 times. Washed samples were dialyzed for 72 h under dark conditions by changing the water every 24 h. Dialyzed samples were centrifuged to remove excess water and later sonicated for 5 minutes to homogenize the samples. Fluorescent images of nanocellulose distributed in hydrogel lenses were obtained using the Nikon eclipse TE2000-U microscope, which was equipped with a camera. Fluorescence was measured using an excitation wavelength of 494 nm and an emission wavelength of 512 nm.

**White light interferometry:** In addition to the optical microscopy of the different hydrogels, a quantitative characterization of the surface structures of the hydrogels was performed using white light interferometry measurements (WLI, NewView7300/ZygoLOT) using 10× and 50× objectives. This results in a range of lateral surface spatial frequencies from 0.002 µm\(^{-1}\) to 2.3 µm\(^{-1}\), which covers the spatial frequency range relevant for light scattering at visible wavelengths. Using the WLI topographic surface data, \(h(x,y)\) two-dimensional isotropic power spectral density (PSD) functions of every sample were calculated for roughness analysis as described in our previous work.\(^{113}\) The rms-roughness \(\sigma\), defined as the standard deviation of the surface heights, can be determined by integrating these PSD functions over the relevant spatial frequency range:

\[
\sigma^2 = 2\pi \int_{f_{\text{min}}}^{f_{\text{max}}} \text{PSD}(f)df
\]

In contrast to simple roughness measurements, this PSD-based procedure allows us to combine different measurements and to retrieve roughness values representative for the entire spatial frequency range relevant for visible light scattering. Moreover, the scattering distribution or angle resolved scattering (ARS) is directly proportional to the surface PSD. For a single sur-
face, which is clean and optically smooth, the Rayleigh-Rice theory predicts:

$$\text{ARS}(\theta_s) = Q \cdot \text{PSD}(f)$$  \hspace{1cm} (2)

The optical factor $Q$ contains information such as the conditions of illumination and observation as well as material properties. In this equation, the spatial frequencies and the scattering angles are related by the grating equation:

$$f = \frac{(\sin \theta_s)}{\lambda}$$  \hspace{1cm} (3)

For visible light applications, assuming $\lambda_{\text{min}}=380$ nm and $\lambda_{\text{max}}=780$ nm and the scattering angles are between 0.1° and 90°, the relevant spatial frequencies for normal incidence consequently range between 0.002 $\mu$m$^{-1}$ and 2.6 $\mu$m$^{-1}$.

5.3.2. Optical characterization

**Light transmittance:** Light transmittance through 1-mm-thick hydrogel strips immersed in a quartz cuvette with deionized water at 25 °C was measured using a UV-Vis spectrophotometer (UV-1650PC, Shimadzu). Transmittance was measured in the range of 200 and 900 nm with deionized water as the reference for 100% transmittance.

**Light scattering:** Angle resolved light scattering measurements were performed to investigate the optical properties of the hydrogel materials. Usually, highly sensitive goniometric scatterometers are used that are based on scanning a detector through an illuminated sample. In this study, a BTDF (Bidirectional Transmittance Distribution Function) Sensor, recently developed at Fraunhofer IOF, was used. In contrast to conventional scatterometers, the BTDF sensor enables scatter measurements within a few seconds and thus allows samples to be measured before drying out. In the setup (Figure 4), a tunable supercontinuum source was used at a wavelength $\lambda$ of 532 nm to illuminate the clamped hydrogel samples at an angle of incidence of $\theta_i=0°$. 
Figure 4. Setup for light scattering measurements of hydrogel samples using the BTDF sensor (reprinted from Paper V).

The wavelength was chosen because it lies in the most relevant part of the spectrum visible to the human eye. A complementary metal-oxide semiconductor (CMOS) detector matrix and a high dynamic range (HDR) procedure were used to measure the scattered light in the transmission hemisphere at a cone angle of 14° around the specular beam.

As the instrument enables rapid 3D scattering measurements, i.e., within a few seconds, 0.3-mm and 5-mm disc-shaped samples of pure PVA, PVA-CNC, and PVA-CNFi were investigated at different stages of drying after removal from the immersion liquid, (water) including almost fully wet conditions.

The 3D scattering distributions were quantified in terms of the angle-resolved scattering (ARS), defined as the power $\Delta P_s$ of the light scattered into the solid angle $\Delta \Omega_s$, normalized to $\Delta \Omega_s$ and the incident light power $P_i$:114-115

\[
ARS(\theta_s) = \frac{\Delta P_s(\theta_s)}{\Delta \Omega_s P_i} \tag{4}
\]

where $\theta_s$ is the scattering angle with respect to the surface normal. ARS is identical to the cosine corrected bidirectional transmittance distribution function, where BTDF=ARS$/\cos(\theta_s)$. Additionally, total forward-scatter (TSf) data were retrieved by integration of the ARS to compare the light scattering properties of the PVA samples. Total forward-scattering (TSf) is defined as the total scattered power $P_s$ in the transmission direction, normalized to the incident power $P_i$.114-115 In the following investigation, total forward-scatter (TSf) will be referred to simply as TS.
5.3.3. Mechanical characterization

**Tensile properties:** Prior to the test, the specimens were kept submerged in water. The samples were tested instantly after being removed from the water to limit the effects of dehydration on the hydrogel. The room temperature was 23 °C (±2 °C). Tensile tests were performed using a laboratory materials testing machine (AGX-series, Shimadzu, Kyoto, Japan). The machine was equipped with a 10-N load cell. The specimens were tested at a cross-head speed of 10 mm/min. A polarized light source of hexagonally arrayed LEDs was used to detect strain-induced birefringence, due to the reorientation of CNC whiskers in the hydrogel during stretching. For polarized light photography, a cross head speed of 50 mm/min was used and polarizers were set parallel and perpendicular to the direction of strain.

**Axial compression:** The samples were tested instantly after being removed from water to limit dehydration of the hydrogel. The room temperature was 23 °C (±2 °C). The tensile tests were performed using a laboratory materials testing machine (AGX-series, Shimadzu, Kyoto, Japan). The cylindrical gel sample of 32 mm diameter and 12.7 mm thickness was set on the lower plate and compressed by the upper plate, which was connected to a 1-kN load cell, at a cross head speed of 10 mm/ min. The samples were compressed to 50% strain to evaluate the performance of the material under demanding conditions.

**Viscoelasticity:** Viscoelastic measurements were performed on a TA Instruments AR2000 Rheometer, using the parallel-plate geometry (diameter 35 mm) frequency sweep mode to measure the storage modulus, $G'$, the loss modulus, $G''$, and the loss tangent, tan δ. The measurements were performed after the hydrogels were dialyzed for 48 hrs. The thickness of the hydrogel sample was 12.7 mm. Strain sweep between 0.01 and 10% at 1 Hz frequency and 25 °C was performed to locate the linear viscoelastic region. Frequency scans between 1 and 100 Hz at 25 °C isothermal conditions were then carried out. The applied strain was 0.25% and the force at the contact between the plate and sample was set to 5 N.

5.3.4. Biocompatibility and other characterizations

**Oxygen Permeability:** The measurements were performed using Ox-Tran 2/21 Mocon instrument equipped with a coulometric detector. The measurements were performed at 37 °C. The relative humidity was maintained between 94 and 100%. Synthetic air (20.9% O₂ and 79.1% N₂) was used throughout the study. The sample was conditioned for 16 hours prior to test outgas dissolved oxygen. The sample was 1 mm in thickness, and the exposure area was 5 cm². The sample was fixed between aluminum masks using
Epiglu™ glue, to avoid leakage. The measurement was performed in duplicate. The underlying principle of the measurement is described in detail in ASTM F1927 - 07 Standard Test Method for Determination of Oxygen Gas Transmission Rate, Permeability and Permeance at Controlled Relative Humidity Through Barrier Materials Using a Coulometric Detector.

**Cell studies:** Human corneal epithelial cells (HCE-2) were selected to study the cytocompatibility of the PVA-CNC hydrogels. The cytocompatibility studies comprised indirect and direct contact tests, together with the evaluation of the cell response when cells were cultured on the hydrogel surface.

HCE-2 cells were purchased from American Type Culture Collection (ATCC) and were cultured in keratinocyte-serum free medium (KSFM) supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, and 5 ng/mL insulin. Cells were grown in a humidified atmosphere at 37 °C, 5% CO₂ and sub-cultured at 80% confluence. Cells were harvested using trypsin treatment and counted using a hemocytometer. Cell viability was assessed using trypan blue staining (90-95% cell viability).

**Indirect cytocompatibility test:** The test was performed in compliance with the ISO 10993-5 procedure. Briefly, the PVA-CNC lens samples were immersed in Dulbecco’s PBS for 24 h and sterilized using UV radiation. The CNC-PVA lenses and Nelfilcon lenses were extracted in culture medium for 24 ± 2 h at 37 °C, 5% CO₂. The surface/volume ratio was 6 cm² mL⁻¹. Cells were suspended in the extract medium at a density of 1×10⁵ cells mL⁻¹ and seeded on a 24-well tissue culture plate (5×10⁴ cells per well). Cells were then incubated for further 24 ± 2 h at 37 °C, 5% CO₂ in a humidified atmosphere. The negative control was the extract medium of tissue culture plate (TCP), and 5% DMSO in culture medium was used as the positive, i.e., to verify that the test can detect a toxic effect. Samples were run in triplicate.

**Cell viability assay:** The Alamar blue (AB) assay was carried out to determine the cell viability. After 24 ± 2-h incubation with the extract medium, cells were carefully rinsed with PBS and 500 μL of AB reagent diluted 1:10 in PBS were added to the wells and incubated for 90 min at 37 °C, 5% CO₂ in a humidified atmosphere. Aliquots of 100 μL from each well were transferred to a black 96 well plate and fluorescence intensity was measured at 560 nm excitation wavelength and 590 nm emission wavelength using a spectrofluorometer (Tecan infinite® M2000). Samples were run in triplicate and the experiment was repeated three times.

**Direct cytocompatibility test:** HCE-2 cell suspensions were prepared in complete KSFM at a density of 1x10⁵ cells/mL and seeded on a 24-well tissue culture plate (5x10⁴ cells per well). Cells were then incubated for 24 ± 2 h at 37 °C, 5% CO₂ in a humidified atmosphere before applying the lens
samples on top of the confluent cell layer. Cells cultured on tissue culture plate (TCP) without the presence of the lenses served as a control (untreated cells). The cells were further incubated for 24 ± 2 h at 37 °C, 5% CO₂ in a humidified atmosphere and thereafter the lens samples were carefully removed. Cell morphology and the integrity of the cell layers were analyzed by light microscopy (Nikon Eclipse TE2000, Japan), while cell metabolic activity was assessed by the AB assay following the protocol described above.

**Cell culture on the hydrogels:** Simulated tear fluid (STF) consisting of lysozyme (2.68 mg/ml); D-glucose (6.50 mg/ml); gamma-globulin (1.34 mg/ml); sodium chloride (6.50 mg/ml); bovine serum albumin (2.68 mg/ml); and calcium chloride dihydrate (0.08 mg/ml) was prepared in deionized water and the pH was adjusted to 7.4 using 0.01 M HCl. The solution was mixed for 30 min using a magnetic stirrer and filtered using 80 µm filter. Lens samples with and without STF pre-conditioning were placed in 24-well tissue culture plates and 5×10⁴ HCE-2 cells suspended in complete KSFM were seeded on top of the lenses (500 μL cell suspension per well) and cultured for 24 ± 2 h at 37 °C, 5% CO₂ in a humidified atmosphere. Cells cultured on TCP served as a control. Samples were run in triplicate and the experiment was repeated three times. Cell response was evaluated by measuring cell viability (AB assay as describe above) and live/dead imaging of adherent cells on the hydrogels.

**Live-dead staining:** Adherent cells were double stained with calcein-AM and propidium iodine (PI) to visualize live and non-viable cells respectively, after 24-h culture. Cell culture medium was removed from the wells, the lenses were carefully rinsed with PBS and 300 μL of assay solution (2 μL calcecin-AM and 1 μL PI per mL of PBS) were added to each sample and incubated for 15 min at 37 °C, 5% CO₂. The stained cells were imaged using a fluorescence microscope (Nikon ECLIPSE TE2000-U) with λex 490 nm, λem 515 nm filter to visualize live cells and λex 535 nm, λem 617 nm filter to visualize cells with compromised membrane integrity.

**Suturing:** The suturing procedure was performed at Griffith’s lab at Linköping University. To determine if implants are amenable to suturing for retention of the implant during surgery, implants were subjected to suture testing. The implants were cut out with a circular trephine to a diameter of 6.75 mm and a circular corneal button of 6.5 mm dimension was removed from an excised porcine cornea to form a wound bed. The implants fit snugly into the wound bed and the placement of 12 interrupted sutures was used to determine the ability of the construct to tolerate suture placement. Implants were scored from “0” to “3”, from no observable effect on the hydrogel to tearing of the hydrogel, respectively, for each suture. Then, a total score for all sutures was summed for each of the triplicate samples. An arbitrary
threshold value for suturability was set at “10” based on the total score for each sample. Anything above the threshold value was deemed fragile for suturing and would therefore require extra care or reinforcement.

**Particle size and zeta potential:** Measurements of the particle hydrodynamic diameter of Chitosan-poly(acryl acid) nanoparticles were achieved via dynamic light scattering (DLS) by using disposable PMMA cuvettes. The ZetaSizer Nano Instrument was used (Malvern Instruments, UK). The measurement angle used for all measurements was 173° backscattering NIBS. Average surface charge of the nanoparticles was measured using a universal dip cell (Malvern Instruments, UK). The same instrument was used as mentioned previously. The zeta potential was obtained from the electrophoretic mobility of the particles using the Smoluchowski model. Each sample, unless mentioned, was equilibrated for 60 s at 25 °C prior to each measurement that was performed in triplicate.

**Fluorescence spectroscopy:** Fluorescence spectroscopy of labeled chitosan-poly(acrylic acid) nanoparticles were carried out on black Corning® 96 well plates (polystyrene, flat bottom wells, 100/cs) with an Infinite M200 Multi-mode Microplate Reader (Tecan, CH). Each well contained a 200 µL sample and each sample was measured once using default settings. λem=520 nm and λex=490 nm.
6. Results and Discussion

6.1 Overview of PVA-CNC hydrogel general characteristics

In this section, a broad outlook on the characteristics of PVA-CNC composite hydrogel, illustrating the key properties crucial for an ophthalmic insert is presented. PVA-CNC hydrogels made with a DMSO to water ratio of 80:20 are discussed here. The results from Paper I show that PVA-CNC hydrogel lenses not only feature desirable optical transparency but also softness, conformity, oxygen permeability, high water content, resistance to mechanical shear, cytocompatibility, and suturability.

Figure 5a shows the AFM image of CNCs used in the reinforcement of PVA hydrogel. Panel b and c in Figure 5 are images of PVA-CNC hydrogel lenses and from this it can be seen that they are transparent, self-standing, and capable of retaining their shape. Figure 5d shows transparency of a 1-mm-thick hydrogel sheet and Figure 5e shows a SEM image of a freeze-dried lens. It can be seen that the hydrogel has an open and macro-porous structure allowing for transport of oxygen as well as other metabolites. Figure 5f shows the PVA-CNC hydrogel implant sutured ex vivo to a porcine excised cornea. The PVA-CNC hydrogel demonstrated excellent suturability by tolerating 12 separate sutures without any tear.

Table 1 presents a summary of physical properties of a number of PVA-based hydrogels, specifically, their optical, mechanical, and oxygen permeability properties. It was observed that the transparency of the PVA-CNC lens was above 95% in the visible range, and moderate UV-absorption properties were also apparent. The PVA-CNC hydrogel exhibited very high water content of 90% or above, which is an important factor in determining the functionality of the contact lens. The refractive index of the material is close to that of distilled water, i.e., 1.33. This implies that the PVA-CNC hydrogel is practically invisible in water. More on optical properties can be found in Papers II & III and will be discussed later. The extra high water content and porous structure of the material provide good oxygen permeability, i.e., $66 \times 10^{-11}$ Dk.

The PVA-CNC hydrogel features a rubber-like mechanical profile, which is unusual for an ophthalmic contact lens or keratoprosthesis material. The stress-strain curve of the PVA-CNC hydrogel resembles that of hyper-elastic materials rather than normal contact lenses (Papers I & IV). Alt-
hough water present in the hydrogel undoubtedly softens the composite, its presence alone cannot explain the observed mechanical profile. Such hyperelastic mechanical behavior is normally detected in collagenous tissues.\textsuperscript{122} This will be dealt in detail in chapter 6.5.

\textbf{Figure 5.} PVA-CNC hydrogel lens: (a) AFM image of carboxylated cellulose nanowhiskers, (b) Self-standing lens, (c) Lens showing conformability, (d) Transparent sheet of hydrogel and (e) SEM image of freeze-dried lens, (f) Picture of PVA-CNC hydrogel implant sutured to ex vivo porcine cornea (reprinted from \textbf{Paper I}).
Table 1. Comparison of the physical properties of lenses based on PVA with typical soft contact lenses (reprinted from Paper 1).

<table>
<thead>
<tr>
<th></th>
<th>PVA-CNC hydrogel</th>
<th>PVA hydrogel, Kita et al.(^{19})</th>
<th>Nelfilcon A, cross-linked modified PVA(^{23})</th>
<th>Typical soft contact lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer content, %</td>
<td>7</td>
<td>25</td>
<td>31</td>
<td>26 - 67</td>
</tr>
<tr>
<td>Water content, %</td>
<td>93</td>
<td>78</td>
<td>69</td>
<td>33 - 74</td>
</tr>
<tr>
<td>Optical properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIS (610 nm), T%</td>
<td>&gt; 95%</td>
<td>&gt; 99%</td>
<td>&gt; 92%</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>UV-A (320 – 400 nm), T%</td>
<td>80</td>
<td>N.A.</td>
<td>N.A.</td>
<td>90</td>
</tr>
<tr>
<td>UV -B (290 – 320 nm), T%</td>
<td>60</td>
<td>N.A.</td>
<td>N.A.</td>
<td>70</td>
</tr>
<tr>
<td>Refractive index</td>
<td>1.33</td>
<td>N.A.</td>
<td>1.38</td>
<td>1.38 – 1.42</td>
</tr>
<tr>
<td>Permeability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(_2) permeability, Dk( (\text{cm}^2/\text{sec}) (\text{mL O}_2/\text{mL x mm Hg}) )</td>
<td>66 (\times 10^{-11})</td>
<td>44 (\times 10^{-11})</td>
<td>26 (\times 10^{-11})</td>
<td>22-130 (\times 10^{-11})</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress to failure, MPa</td>
<td>0.15±0.02</td>
<td>1.2</td>
<td>N.A.</td>
<td>0.4-3.0</td>
</tr>
<tr>
<td>Strain to failure, %</td>
<td>383±15</td>
<td>500</td>
<td>N.A.</td>
<td>178-245</td>
</tr>
<tr>
<td>Toe point Modulus, MPa</td>
<td>0.016± 0.003</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Young’s modulus, MPa</td>
<td>0.071± 0.007</td>
<td>4.61</td>
<td>N.A.</td>
<td>0.3-1.6</td>
</tr>
</tbody>
</table>
Furthermore, viscoelasticity experimental results indicated that the composite hydrogels have a true gel behavior. The viscoelastic moduli, G’ and G’’, were well separated and parallel to each other at the tested frequencies between 1 and 20 Hz, suggesting true viscoelastic solid behavior. In this region, the overall system is characterized by a damping factor δ as low as ~1.5°, which is remarkable, given that its water content is above 90% (Papers I & V).

Figure 6. Cell viability of human corneal epithelial cells (HCE-2) cultured in extract medium of PVA-CNC hydrogel in the presence of 5% DMSO (positive control). The data are expressed as percentage of the negative control (tissue culture plate extract medium) and represent the mean ± SEM (*** p < 0.01)). Cell viability values greater than 70% of the negative control indicate a non-cytotoxic response (reprinted from Paper I).

The indirect cytocompatibility test showed that HCE-2 cells cultured with the extract medium of PVA-CNC hydrogels exhibited viability well above the toxicity limit of 70% as defined by ISO standard 10993:5. Thus, indicating that toxic effects due to leaching of any residual chemicals in the hydrogel were not detected.

Furthermore, when HCE-2 cells were cultured on the hydrogel surface (Papers I & VI) it was shown that the hydrogel material supports cell adhesion and cell proliferation. A more detailed account of the observations presented in this section will be discussed in the forthcoming chapters.
6.2 Gelation mechanism

When a homogenous PVA solution is quenched from a high temperature to -20 °C, gelling occurs by the physical cross-linking of PVA chains. It can be seen from Figure 7 that PVA solutions did not freeze at the operating cryogelation temperature, i.e., around -20 °C. This gelation step was found to influence optical, mechanical and viscoelastic properties of the hydrogel (Papers II, IV & V). In order to explain the mechanical and optical properties of PVA hydrogels, it is important to understand the gelation mechanism in the DMSO-water mixed solvent system.

Though the DSC curve in Figure 7 shows that no solvent composition resulted in freezing around -20 °C, practical observation showed that PVA solution made from a DMSO to water ratio of 90:10 did freeze. Previously, it was known that DMSO-water systems show a sharp decrease in freezing point. Nevertheless, maximum decrease in freezing point was reported to be around 60:40 composition and 90:10 composition freezes at around 0 °C, whereas 80:20 composition is on the border line of phase transition around -30 °C.

The curves we observe in Figure 7 may be a result of kinetic effect due to slow cooling rate, i.e. 10 K/min. Based on this it can be said that 90:10 compositions may have low crosslinking due to ice formation and 60:40 compositions may have greater degree of crosslinking due to higher mobility of PVA chains. The 70:30 and 80:20 compositions fall in between these extremes and may follow a logical trend. This crosslinking during gelation alters the properties of the hydrogel due to structural variations.

Figure 7. DSC thermogram showing the freezing of PVA solutions (adapted from Paper V).
Introduction of CNCs in the PVA solutions altered the freezing and thawing temperatures of the solutions and there by affecting the structure, due to change in crosslinking pattern (see Paper V). CNCs being charged and highly hydrophilic might have had an effect on the DMSO-water interactions. Properties of hydrogels made with a DMSO to water ratio of 80:20 will be discussed in the following chapters unless otherwise specified.
6.3 Nanocellulose-reinforced PVA hydrogel structure

In order to visualize the internal structure of the contact lenses, they were gently freeze dried to avoid shrinkage and investigated using SEM imaging. It can be seen from Figure 8 that the composite formed an open, interconnected, continuous, and macroporous honeycomb-like structure. In Figure 8a, small projections are distinctly observed stretching outwards from the matrix cell walls. It is plausible that these projections are CNC whiskers. In Figure 8b, no such projections could be visualized; instead, occasionally long filaments could be seen extending over several cells.

*Figure 8. (a) SEM image of freeze dried contact lens from PVA-CNC, (b) SEM image of freeze dried contact lens from PVA-CNF (adapted from Paper II).*
Figure 9 shows the optical microscopy images of the hydrogels in a wet state. The pore size was slightly different among hydrogels of different composition owing to nanostructural interactions between the PVA matrix and nanocellulose moieties. The dark patches causing light occlusion in the PVA-CNF hydrogel are possibly due to CNF aggregates present in planes that are lower than the plane of imaging. The formation of CNF aggregates is due to its high viscosity, which in turn is due to the high aspect ratio of CNF. As expected optical microscopy did not shed any light on nanocellulose distribution, as the resolution of the technique is limited.

![Figure 9. Optical light micrographs of (a) pure PVA, (b) PVA-CNC and (c) PVA-CN hydrogels (reprinted from Paper III).](image)

To obtain more information about the distribution of nanocellulose in the hydrogel matrix, fluorescence microscopy technique was used. Figure 10 shows the fluorescence microscopy images of hydrogels where nanocellulose components were labelled with fluorophore groups, i.e., DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein). While it was not expected that fluorescence imaging would reveal details on individual nanocellulose units, due to the low resolution of the technique, it still provided some information about the distribution of nanocellulose inside the hydrogel. It can be seen that CNCs are distributed much more homogeneously than CNF, which tends to
form lumps. As expected, the control pure PVA hydrogel showed negligible fluorescence.

Figure 10. Fluorescence micrographs of (a) pure PVA, (b) PVA-CNC and (c) PVA-CNf hydrogels (reprinted from Paper III).

The surface properties of the hydrogel in their native state determine several functional outcomes, as it is the point of contact with both the eye and atmosphere. The surface morphology of the hydrogels was evaluated using the AFM technique. Figure 11 shows the AFM micrographs of the hydrogels in a wet state, i.e., immersed in water. The surface topography of pure PVA samples exhibit a wavy pattern of moderate ruggedness. The surface topography of PVA-CNC samples appears rougher, when compared to pure PVA samples. In PVA-CNf samples the surface topography is also rougher compared to pure PVA samples.
Overall, it can be said that the composite hydrogel has a well-connected open macroporous network structure, with rough surface morphology. The distribution of CNCs in the PVA matrix is fairly uniform, whereas CNFs form bundles and their distribution is non-homogeneous. The structural aspects of the material are significant as they form the base to discuss several other properties.

6.4 Optical properties

6.4.1. Transparency

Transparency is an important factor in determining the performance of ophthalmic materials. The transparency of 1-mm-thick hydrogel samples were measured using a UV-vis spectrophotometer. It can be seen from Figure 12 that all of the samples have high transparency, i.e., ≥ 90% in the visible light spectrum (400 nm – 700 nm). The high transparency is related to the exceptionally high water content and structure of the hydrogel. The nanocellulose reinforcement effect on transparency in the visible region is not significant and is more pronounced in CNF reinforced samples, than those reinforced with CNC. The decrease in transparency of CNF samples may be related to the formation of aggregates and their non-uniform distribution as discussed previously. Furthermore, the decrease in transparency may also be due to the
higher aspect ratio of CNF and hence, stronger light scattering. This is contrary to the CNC samples that have a smaller aspect ratio and less scattering.

Figure 12. Comparative UV-Vis transmission spectra of 1 mm hydrogels with 10% PVA and 1% CNF/CNC (reprinted from Paper II).

The other interesting aspect of the hydrogel material is the UV light blocking properties of PVA, as manifested by a sharp drop in transparency in the region between 200 nm and 400 nm. Considering the detrimental effects of UV light on the eyes like pterygium and cataract, the intrinsic UV blocking properties of PVA-based hydrogels are highly beneficial for ophthalmic applications.17,124

6.4.2. Light scattering

Scattering is an important and complex phenomenon that affects the visual acuity of ophthalmic devices. Total scattering can be classified into surface and bulk scattering. Surface scattering is primarily due to surface topography and surface chemistry of the material. Bulk scattering is a result of internal structure and composition of the material and varies with thickness of the material. The 3D angle resolved scattering (ARS) technique coupled with white light interferometry (WLI) was used to understand the factors affecting the scattering in hydrogel samples. The scattering values at different time intervals and corresponding roughness values are shown in Figure 13a. It is evident that scattering is influenced by roughness, which is a result of the
drying of the surface water film of the hydrogel. Rapid (within minutes) drying and shrinking of hydrogel-based contact lenses is normal and has been previously described in the literature (under physiological conditions the tear fluid film is replenished through blinking).\textsuperscript{125}

\textit{Figure 13.} (a) 3D ARS and WLI measurements and (b) 1D ARS for the thin PVA-CNC sample at different drying states (reprinted from Paper III).

It was observed that after 10 min in air, the sample already becomes rigid. Consequently, no further change in surface roughness or scattering with increased drying time was observed until the sample loses its form and becomes strongly wrinkled. The WLI roughness measurements in Figure 13a and 1D ARS data from Figure 13b confirm the visual observations.
In order to separate the bulk and surface factors contributing to scattering, thin and thick samples were analyzed. The scattering data of the 0.3-mm-thick samples and 5-mm-thick samples are shown in Figure 14. The total scattering (TS) values calculated from the ARS are also provided in the respective images. The PVA-CNF sample has higher bulk scattering when compared to both pure PVA and PVA-CNC samples. This behavior is assumed to result from CNF aggregates in the bulk of the hydrogel material as observed in Figures 9 & 10.

6.5 Mechanical properties

6.5.1. Effect of nanocellulose on tensile strength

As hydrogels are inherently weak, nanocellulose was used to enhance the mechanical properties. Figure 15 illustrates the effect of the nanocellulose aspect ratio on PVA hydrogel mechanical properties. For clarity and as a result of better optical properties, samples with a DMSO:water ratio of 80:20 are discussed, while values for tensile strength, elongation, and modulus for all other sample compositions can be found in Paper IV. It is seen from Figure 15 that the hydrogels exhibit the stress-strain curves characteristic of hyperelastic materials, i.e., high elongation at low stresses and gradual stiffening with further applied stress. Such behavior is characteristic of collagen-based soft tissues and is normally difficult to mimic in synthetic materials. It is evident from Figure 15 that the inclusion of CNC and CNF strongly affects the mechanical properties of the PVA hydrogel. Almost a three-fold
increase in tensile strength of the PVA-CNF 80:20 sample was recorded compared to the pure PVA 80:20 hydrogel.

The reinforcing effects of CNF and CNC were different. With CNF reinforcement, the samples become much stronger than pure PVA but also less elastic. When loaded with CNC, both maximum stress and elongation increased when compared to pure PVA hydrogel. This difference in behavior could be attributed to the aspect ratio of CNC and CNF. The results also suggest a substantial degree of entanglement between nanocellulose and PVA, probably due to hydrogen bonding.

![Graph showing the effect of nanocellulose reinforcement on the tensile stress-strain relationship of the composite hydrogel with a solvent composition of DMSO:water of 80:20.](adapted from Paper IV)

6.5.2. Mechanism of strain-induced stiffening

The stress-strain curves of the hydrogels showed a behavior that is typically observed in collagenous fibrous tissues such as tendons, ligaments, and cornea.\(^{122, 126-128}\) For a biomaterial to mimic the mechanical behavior of a soft tissue, it is important to have a solid phase that combines both soft and rigid regions. While it can be argued that pure PVA hydrogel possesses both rigid crystallites and soft randomly coiled chains, the effect of the reinforcement is much clearer when another component of higher rigidity is introduced.
The stress-strain curve begins with a toe region where the soft PVA chain network begins to unfold and align in the direction of loading. Since it is easier to stretch out a soft, coiled PVA chain network, this part of the stress-strain curve shows relatively low stiffness. As the PVA chains straighten and rigid components realign, the material gradually stiffens under an applied tensile load. Eventually, as the individual polymer chains within the network begin to fail, damage accumulates, and the sample finally ruptures. Thus, the overall behavior of the hydrogel depends on the network structure, which in turn depends on the cross-linking density, the stiffness of the reinforcing component, and its aspect ratio.

The fiber reorientation behavior at high strains was verified experimentally using strain-induced birefringence experiments. When the hydrogel with anisotropic distribution of crystalline domains, i.e., either nanocellulose or PVA crystallites, is illuminated by polarized light in the relaxed state, it appears dark. As the sample is pulled and the rigid components realign, the sample appears progressively bright when observed between cross-polarizers, which is a phenomenon known as birefringence. Figure 16 shows a typical strain-induced birefringence sequence for PVA-CNF hydrogel. A similar birefringence behavior was observed in polyamide hydrogels reinforced with clay nanoparticles.\textsuperscript{129-130} The implications of the reorientation of reinforcing components in a hydrogel are further explored through modeling. Furthermore, the micromechanics model, developed to predict the mechanical behavior of the composite hydrogel, fits the expected curve based on experimental data. The model is presented in detail in Paper IV.
Figure 16. Strain-induced birefringence curves for a PVA-CNF hydrogel. Time-lapse images of the PVA-CNF hydrogel (a-f) illuminated by a polarized light source. Pulling was set to 50 mm/min, and the increase in CNF alignment along the direction of loading is indicated by increased brightness in polarized light (reprinted from Paper IV).
6.5.3. Effect of CNCs on compressive strength

The hydrogel samples were further subjected to a compressive strength test at 50% strain. Figures 17a and 17b show the curves from the compression tests of pure PVA and PVA-CNC hydrogels synthesized from different solvent compositions. The solvent effect is clearly visible in pure PVA hydrogels (Figure 17a), whereas in PVA-CNC hydrogels (Figure 17b), the solvent effect is nullified. It was observed previously (Figure 16 & Paper I) that during elongation, stiffening occurs by strain induced alignment of the reinforcing elements along the direction of stress. In this context, considering the structure of the hydrogels (Figures 8 & 9), it can be said that the stiffening occurs due to strain induced alignment of CNCs perpendicular to the direction of stress during compression.

![Graph showing stress vs strain for different PVA solvent compositions](image)
6.5.4. Viscoelasticity

The viscoelastic properties of the hydrogel are of interest as it exhibits soft tissue like behavior. The viscoelastic behavior of the hydrogel can be described by an elastic (storage) modulus $G'$, which reflects the reversibly stored energy, and a viscous (loss) modulus $G''$, which reflects the irreversible energy loss. It can be seen in Figure 18a that the system is characterized by an exceptionally high elastic modulus $G' (>15 \text{ kPa})$, and a viscous modulus $G'' (>400 \text{ Pa})$ for a material with water content above 90%. The moduli are well separated and are almost parallel to each other at the tested frequencies between 1 and 20 Hz; this suggests true viscoelastic solid behavior.

The damping factor $\tan \delta$ is defined as the ratio between loss modulus ($G''$) and elastic modulus ($G'$). Damping is the dissipation of energy in a material under cyclic load. It is a measure of how well a material can get rid of the absorbed energy and is reported as the tangent of the phase angle ($\delta$) between an impact force and the resultant force that is transmitted to the matrix network. In the lower frequency region, i.e. 5 Hz, the overall system is characterized by a damping factor ($\delta$) as low as $\sim 1.5^\circ$. A damping factor ($\delta$) of $10^\circ$ or lower suggests true viscoelastic behavior. A slight increase in
the value of the damping factor (δ) was observed for the sample produced from DMSO:water ratio of 60:40 mixture compared to the other samples, (Figure 18b).

The hydrogel with a 60:40 solvent composition also exhibited a higher loss modulus (G") compared to other samples. This could be due to bigger PVA crystallites formed during gelation as discussed in chapter 6.2. More detailed information can be found in Paper V.

Figure 18. Viscoelastic properties of PVA-CNC hydrogels with different DMSO:water ratios. (a) Dynamic viscoelastic moduli G', G" of PVA-CNC samples and (b) delta as a function of solvent composition (adapted from Paper V).
6.6 *In vitro* cytocompatibility

The PVA-CNC sample synthesized with a DMSO:water ratio of 80:20 was chosen for further cytocompatibility evaluation. The response of HCE-2 cells to direct contact with the hydrogel material was evaluated by placing the lenses on top of confluent cell layers and determining cell viability, morphology and the integrity of the cell layer after removal of the samples. As a comparison, a PVA-based commercially available lens, i.e. Nelfilcon A, was also tested. The cell metabolic activity after 24-h exposure to PVA-CNC and Nelfilcon A lens is presented in Figure 19. It can be seen from Figure 19a that metabolic activity of the cells cultured on the lenses is similar to that of the control (untreated cells).

![Figure 19. HCE-2 cells after 24 h direct contact with the PVA-CNC hydrogels and following removal of the materials. a) cell metabolic activity assessed by the alamar blue assay. Data represent the mean ± standard error of the mean for n = 3. One way ANOVA was applied. No significant difference was observed between cells exposed to the hydrogels and the control (untreated cells). b-d) representative light microscopy images of the cell layers after direct contact with b) PVA-CNC and c) Nelfilcon A; while d) correspond to untreated cells. The scale bar corresponds to 100 µm (reprinted from Paper VI).](image)

The morphology of the adhered cells after direct contact with the hydro-gels was evaluated using optical microscopy. It can be seen from Figure 19b that the cells exposed to the PVA-CNC lens present morphology similar to that of the cells in contact with Nelfilcon A (panel c) and the control (panel d). Besides, the integrity of the cell layer was not affected by the contact with
the PVA-CNC hydrogel. These results showed that the physical presence of the lenses does not affect corneal epithelial cell monolayers and further confirm the non-toxic profile of the PVA-CNC hydrogels described by the indirect test.

The PVA-CNC lens material was subjected to a further test to investigate the lens-cell interactions. HCE-2 cells were seeded onto the lenses after exposing them to STF for 24 h, i.e. STF pre-conditioned lenses, and cultured for 24 hours. A similar set of experiments was carried out in parallel, without exposing the lenses to STF.

The cell metabolic activity results are depicted in Figure 20, and they indicated a significant difference between the number of metabolically active cells on the surface of the hydrogels compared to the control (cells cultured on TCP). However, such differences represent only 30 %, thus relatively good attachment and viability of the cells on the studied materials can be claimed.

![Figure 20](image)

*Figure 20.* Cell viability of HCE-2 cells cultured on PVA-CNC lens and tissue culture plate (control) for 24 h, with and without STF pre-conditioning of the material surfaces (STF and No STF respectively). Data represent the mean ± standard error of the mean for n = 3. Statistical significant differences between the PVA-CNC and the controls are indicated with ** *p* < 0.01 and *** *p* < 0.001 by two-way ANOVA (reprinted from *Paper VI*).

The live/dead staining fluorescent images of adherent cells confirm that the PVA-CNC hydrogels support corneal epithelial cell attachment, with cells displaying the typical morphology of the epithelial cells and a few number of non-viable cells (i.e. cells with compromised membrane integrity), comparable to the cells cultured on the TCP (Figure 21).
Besides, these live/dead assay results are in agreement with the previous findings where corneal epithelial cells labelled with green fluorescent protein (GFP) were cultured on PVA-CNC hydrogel material (see Paper I).

The effect of STF pre-conditioning of the lens surface in cell attachment and viability was investigated. A formation of a tear film on the surface of the ophthalmic material is expected upon contact between the material and tears. The presence of the protein film is anticipated to influence the interactions at the material surface, including those with corneal epithelial cells. Interestingly, no significant differences between the STF and no STF experiments were found (Figure 20 and 21).
Figure 21. Live/dead staining of HCE-2 cells cultured on PVA-CNC lens and tissue culture plate (TCP) for 24 h. Panels (a, b) and (c, d) show images of live (green) and dead cells (red), respectively, in surfaces pre-conditioned with simulated tear fluid, while panels (e, f) and (g, h) show images of live (green) and dead cells (red) respectively in materials not previously exposed to STF. The scale bar corresponds to 100 µm (reprinted from Paper VI).
6.7 Controlled drug delivery

The pure PVA and PVA-CNC hydrogels prepared using a DMSO to water ratio of 80:20 were tested as potential controlled ophthalmic drug delivery vehicles. Chitosan-poly(acrylic acid) nanoparticles were used as model drug carriers and were integrated into the hydrogel lens. A controlled extended release can be achieved by enzymatic degradation of the nanoparticles in presence of lysozyme, an enzyme present in tear fluid. To test the practicality of the concept, chitosan-poly(acrylic acid) nanoparticles were subjected to degradation in the presence of lysozyme. From the SEM images in Figure 22, it can be seen that the nanoparticles were completely degraded by lysozyme after a 24-h incubation period at room temperature.

![Figure 22. SEM image of a freeze-dried sample containing chitosan-poly(acrylic acid) nanoparticles in (a) deionized water at pH 5.8 after approximately 24-h incubation at room temperature, (b) 0.2 mM lysozyme solution at a pH of ~5.8 after approximately 24-h incubation at room temperature (adapted from Paper VII).](image)

The formation of the aggregates is probably due to the sample preparation procedure. Each particle has a diameter of approximately 200–500 nm and net positive surface charge of 10 ± 5 mV. The nanoparticles that were incubated in 0.2 mM lysozyme for 24 h formed flaky sheets, as shown in Figure 22b. The absence of any granular formations or spheres in the sample confirms the hypothesis, that lysozyme has indeed hydrolyzed the chitosan and disintegrated the nanoparticles.

The stability of the nanoparticles during the hydrogel synthesis conditions is important for their successful integration into the hydrogel matrix. To determine this, the nanoparticle suspension was heated for an hour in a DMSO-water (80:20) solution at 120 °C. In the next step, the solution was first cooled to 60 °C and subsequently to 25 °C. Particle size measurements were made at both these time points. The procedure was carried out again to see the impact of repeated heating on particle stability. The change in particle diameter was used to determine the physical stability of the nanoparticles. From Figure 23, it can be observed that there was a significant decrease in diameter after the first cycle of heating, which can most likely be due to
the dehydration of the nanoparticles. No significant change in diameter was observed between first and second cycle heating. It can be said that the chitosan-poly(acrylic acid) nanoparticles can withstand the hydrogel processing conditions but with an initial drop in the diameter.

![Diagram](image)

**Figure 23.** Diagram of how the average hydrodynamic diameter of the nanoparticles is affected by two cycles of heating to 120 °C for 1 h and cooling in a mixed DMSO and deionized water solution (80:20). Each bar represents the average of three measurements and each error bar the average sample standard deviation (reprinted from Paper VII).

After ascertaining the stability of nanoparticles, they were fluorescently labelled to study their distribution and release behavior in the lenses. FITC-labeled nanoparticle loaded hydrogels were prepared by either mixing the nanoparticle suspension in a PVA solution or mixing the suspension with a CNC suspension prior to its addition to the PVA solution. Upon addition of the nanoparticle suspension to the CNC suspension, an immediate in situ gelation occurred. The nanoparticles carry a net positive charge and it is possible that the ionic interaction between the nanoparticles and CNCs resulted in the gelation.

The addition of labeled nanoparticle suspension to a PVA solution resulted in a clear yellow-tinged solution, while the one prepared from the gelled nanoparticle and CNC suspension on the contrary, resulted in a lumpy yellow solution. Upon casting and freeze-thawing of the hydrogels the resulting NP-PVA lenses turned clear and transparent, while the NP-PVA-CNC lenses had a non-homogeneously spotted appearance as shown in Figure 24. Be-
cause of the high viscosity of the NP-CNC gel, the distribution of the nanoparticles in the lenses was highly varied, leading to both larger and smaller gelled aggregates clearly visible to the naked eye.

Figure 24. FITC-labeled chitosan-poly(acrylic acid) nanoparticles in the left image: a polyvinyl alcohol hydrogel lens and, in the right image: a cellulose nanocrystal reinforced polyvinyl alcohol hydrogel lens (reprinted from Paper VII).

In order to model the release behavior of the lenses, the fluorescence intensities of 10 mM and 0.2 mM lysozyme saline solutions containing NP-PVA and NP-CNC-PVA lenses were measured over a 28-h period. It can be seen from Figure 25 that negligible amounts of FITC-labeled nanoparticles/chitosan fragments were released during the first two hours in all solutions. An increased fluorescence intensity of the PVA lens in lysozyme was observed between 5-10 hours. The fluorescence in the saline solution containing the NP-PVA lens also showed an increase in intensity during the same time period, suggesting that the particles are leaching out of the lens as a result of hydrogel swelling. The slow diffusion mediated transport of the nanoparticles during the first 10 h, as noted, can be attributed to the slow equilibration of the gel from the influx of electrolytes into the network. The slower release which is observed for the lens in lysozyme compared to the lens in saline solution is noteworthy. The delayed release appears to be due to the enzyme adsorbing to the surface of the lens and acting as a diffusion barrier. For the PVA-CNC lenses, the fluorescence intensity over the inspected time interval shows little difference in two solutions. The intensity fluctuates around the base value, which suggests that gelation effectively prevents the leaching of nanoparticles from the lens.
Figure 25. Release of FITC-labeled chitosan fragments/labeled nanoparticles from PVA lenses and PVA-CNC lenses that have been incubated in 0.2 mM lysozyme and 10 mM saline solution without lysozyme for 28 h at room temperature. Each point represents the average of five measurements and each error bar the average sample standard deviation (reprinted from Paper VII).

In order to confirm the presence of the labeled nanoparticles within the NP-PVA hydrogel lenses and to also better visualize the particle distribution in both lens types, fluorescence imaging was employed, as shown in Figure 26. A nanoparticle cluster between 200 and 400 µm can be observed to be present in the NP-PVA lens before its exposure to lysozyme. This seems to indicate that the nanoparticles aggregate a little during the process of integration into the hydrogel. The clusters also seem to be homogeneously distributed within the lens, whereas the clusters in the NP-PVA-CNC lens appear to be much less homogenous.

It can be seen from Figure 26 that there were no nanoparticle clusters present in the PVA lens after incubation in lysozyme solution. In the case of the PVA-CNC lens a diffused cloudy mass was observed. This seems to support the previous observation that nanoparticles do not leach out from the in-situ gel formed during the loading of nanoparticles into the lens. The lower fluorescent intensity also indicates that the lysozyme seems to be able to disrupt these interactions to some degree, perhaps by binding to the CNC, which could enable the enzyme to hydrolyze chitosan on the nanoparticles that are located towards the surface of the lens.
Figure 26. Fluorescence image showing the action of lysozyme on FITC-labeled chitosan-poly(acrylic acid) nanoparticles loaded into the contact lens after 30 h of incubation (adapted from Paper VII).
7. Conclusions

Nanocellulose-reinforced PVA hydrogels intended for ophthalmic applications were presented in this thesis. The results were discussed in terms of structural, optical, mechanical, cytocompatibility, and drug delivery properties. The main advantages were found to be high water content, oxygen permeability, transparency, macroporosity, suturability, elasticity, softness, ability to block UV-light, controlled drug delivery, and cytocompatibility.

In this thesis, it was found that transparent nanocellulose can be introduced into PVA hydrogels by a simple blending procedure. This allows the production of transparent self-standing hydrogels with mild UV-blocking properties. The hydrogels feature a macroporous structure filled with water that plays a crucial role in comfort, oxygen transport and biocompatibility. It was observed that the aspect ratio of nanocellulose plays an important role in influencing the morphology, transparency, and light scattering properties of the hydrogel. CNF reinforced hydrogels showed higher scattering and lower transparency when compared to CNC reinforced hydrogels due to the aggregation of high aspect ratio nanofibrils. Furthermore, it was found that the solvent composition used in hydrogel synthesis plays an important role in determining the structural and optical properties of the lenses. Hydrogels made with a DMSO:water ratio of 80:20 were found to possess optimum transparency under experimental conditions.

The composite hydrogels exhibited a collagen-like strain-induced stiffening behavior due to re-orientation of the reinforcing elements in the direction of strain. Mechanical characterization of the hydrogel samples indicated that CNC and CNF have different reinforcement effects on tensile properties. CNF primarily enhanced the tensile strength and reduced elasticity, whereas CNC enhanced the tensile strength and elasticity. The results showed that the mechanical properties were controlled by parameters such as composition of the gelling solvent and nanocellulose aspect ratio. The results of this study suggest that soft-tissue-like mechanical properties require high water content and a specific type of solid matrix that is also simultaneously composed of soft and rigid domains.

Based on the results from measurements of optical and mechanical properties, the PVA-CNC hydrogels synthesized with a DMSO:water ratio of 80:20 were selected for cytocompatibility analysis. The results of cell studies from both indirect and direct contact studies indicate that the hydrogels are cytocompatible towards corneal epithelial cells. Furthermore, it was shown
that the hydrogels support corneal cell attachment. The PVA-CNC hydrogel also exhibited a good suturability score and was stable when sutured ex vivo to a porcine excised cornea.

Chitosan-poly(acrylic acid) nanoparticles loaded lenses were developed to test the concept of enzyme triggered ophthalmic drug delivery. The results indicate that the nanoparticles were totally degraded by lysozyme. Pure PVA lenses indicate a quicker release rate of nanoparticles over a 28-h period, whereas the PVA-CNC lens showed a slow and steady release rate. This model drug delivery platform, in the form of nanoparticle-loaded lenses, presents a promising opportunity to address the issue of the low bioavailability of drugs in ophthalmic drug delivery systems.

Overall, the PVA-CNC-based hydrogel appears to be a promising material for ophthalmic applications. Further studies are needed to evaluate the hydrogels in vivo. Further experiments shall also include signaling peptides to enable true cornea regeneration application. Identifying suitable drugs that can be loaded into chitosan-poly(acrylic acid) nanoparticles and investigating their release profile will be a logical step in bringing the material closer to practical applications.
Sammanfattning på svenska

Hornhinnan är en transparent vävnad som utgör ögats yta. Ljus bryts vid hornhinnans yta och fokuseras på näthinnan med hjälp av en självjusterande lins belägen bakom hornhinnan. Förmågan att se skarpt beror på den optiska klarheten av denna transparenta vävnad. Skadad hornhinna som konsekvens av en olycka eller infektion leder till försämrad syn och kan i vissa fall leda till blindhet om lämnad okorrigerad. Världshälsoorganisationen uppskattar att 50 miljoner människor i världen är blinda och att skada eller sjukdom som påverkar hornhinnan är de främsta orsakerna efter grå starr.


Förutom korneal blindhet är okulär administration av medicin den andra stora oftalmologiska utmaningen som vården står inför. Ögondroppar i form av suspensioner och lösningar är den vanligaste metoden för administrering av läkemedel till ögat. Den låga biotillgängligheten för läkemedel som administreras via denna metod är dock en stor nackdel. Idag är topikal läkemedelsadministration och intravitreal injektion vanliga behandlingsmetoder. Även implantat för att uppnå bättre leverans av läkemedlet förekommer. Dessa metoder är dock invasiva och medför i många fall komplikationer. Idealt skulle en läkemedelsfrisättande mjuk kontakttins kunna hjälpa för att möta den terapeutiska utmaningen som uppstår på grund av låg biotillgänglighet av läkemedlet. Mjuka kontaktlinser tillverkas vanligtvis av hydrogeler eller silikon.

Hydrogeler är vanligt förekommande material inom okulär vård och används för kontaktlinser, bandagelinser, hornhinnetransplantationer och som stödstruktur för egen vävnadstillväxt. Flertalet syntetiska och biopolymerba-
serade hydrogeler undersöks inom det oftalmologiska vetenskapsområdet. Poly(vinylalkohol) (PVA) är en sådan syntetisk polymer som sedan länge är känd och använd inom den oftalmologiska vetenskapen. PVA-baserade hydrogeler har hög potential för användandet som terapeutisk kontaktlinss och kan även bli ett alternativ till hornhinnetransplantation.

Hydrogeler är i sin natur mekaniskt svaga på grund av deras höga vatteninnehåll och kräver oftast tvärbinding av polymeren eller annan typ av förstärkning. I den här avhandlingen förstärktes PVA med nanocellulosa för att framställa strukturellt stabila hydrogeler. Ett DMSO-vatten-baserat upplösningssystem följt av kryogeleringsteknik användes för att erhålla transparenta hydrogeler. Egenskaperna av dessa hydrogeler analyserades för att undersöka möjligheten att applicera dessa inom områden rörande okulär läkemedelsleverans och regenererande hornhinneimplantat.


Genom mekanisk analys upptäcktes att hydrogeler påvisade en stress-inducerad styvning på grund av linjering av de förstärkande elementen i den externa kraftens riktning. Denna typ av beteende associeras normalt med hyperelastiska material och mjuka vävnader. Vidare visade det sig att förstärkning med nanokristallin cellulosa och nanofibrillerad cellulosa påverkade tänjarbarheten olika. Nanofibrillerad cellulosa ökade den mekaniska styrkan men reducerade elasticiteten, medan nanokristallin cellulosa förbättrade både styrka och elasticitet. Resultaten av denna studie visar att materialet bör bestå av högt vatteninnehåll och en specifik strukturl arkitktur som kombinerar mjuka och stela byggenar för att uppnå likhet med mänsklig vävnad. Lösningsmedlets komposition under syntesen konstaterades ha en betydande roll för de strukturella egenskaperna hos hydrogelererna, som i sin tur påverkar tänjarbarheten samt de optiska, viskoelastiska och mekaniska egenskaperna.

Inga toxiska effekter mot korneala celler påvisades under biokompatibilitetsstudier av komposithydrogeler bestående av PVA och nanokristallin cellulosa. Ytterligare cellstudier indikerade att korneala celler påvisade en snabb och gynnsam tillväxt på hydrogelernas yta. Ex vivo studier demonstrerade vidare hydrogeleras tillförlitliga mekaniska styrka efter att de blivit påsydda på hornhinna från gris.
Acknowledgements

First, I would like to express my sincere gratitude to Prof. Albert Mihryan for giving the opportunity to work with complete freedom and being the constant source of encouragement and inspiration. He is greatly acknowledged for his patient supervision and scholarship that have shaped the direction of this work. His guidance has helped me during the good and bad times of research process and writing of this thesis. I could not have envisaged having a better supervisor and mentor for my doctoral studies.

I would like to thank my co-supervisors Natalia Ferraz, Cecilia Persson and Jonas Lindh for helping me with the experiments and for their insightful comments during discussions. This work couldn’t have been possible without your guidance and support.

I had the pleasure of having successful collaborations during my PhD studies and I would like to extend my gratitude to all my co-authors, with special thanks to Prof. May Griffith, Prof. Sven Schröder, Dr. Thomas Joffre, Michelle Åhlen, Dr. Nadja Felde, Dr. Viviana Lopes, and Dr. Ramiro Rojas.

I would like to thank Ingrid, Jonatan, Maria Skoglund, Maria Melin, Per-Richard, Mikael, and Enrique for their assistance with administration and computers. I would like to thank Victoria, Fredric, and Jan-Åke for their technical support in the cleanroom.

I am thankful to my current and former colleagues at the NFM group for maintaining a vibrant environment for research and for the informal discussions we had. I would like to thank Prof. Maria Stromme for being a continuous source of inspiration to the group, with her positive energy and enthusiasm for science. I would like to thank Prof. Martin Sjödin and Prof. Ken Welch for their efforts in ensuring that the lab is functional and in order.

I would like to thank Petter for sharing office and helping me with practicalities in the lab during my early days. It always feels good to have a conversation with you. I would like to thank Christoffer for his kindness and openness in allowing me to rent his place, without which my life would have been terrible in the dreadful rental market of Uppsala. I am thankful to Simon, a good friend, officemate, travel companion and a co-author. Thank you for those priceless AFM images and wonderful illustrations. I will always remember the days we spent during the ACS conference at San Diego. The cheese cake factory and the tacos experience is a memorable one. I would like to thank Alex for his generosity in translating the thesis summary.
to Swedish. It is always fun to have a conversation with you about our projects and the papers we were working on. I would like to thank Levon, my other officemate for his insights on biology related projects and pastime conversations during random breaks. That mitochondria thing might work.

I would like to thank Tao for helping me during my days in Luleå, Chang-Qing for teaching me several lab techniques in cellulose analysis and Kai for sharing cell study protocol. I would like to thank my other colleagues Maria, Mia, Lisa, Teresa, Sara, Celina, Cecilia, Olof, Christian, Igor, Chao, Li, Peng, Hao, Rikard, Daniel, Ocean, Jiaojiao, Shengyang, Changgang, Pengfei, Jun, Rui, Huan, and Lulu. It has been a great pleasure working with all of you. Thank you for being kind and friendly to me. Many thanks to the cell lab members: Gry, Shiuli, Michael, Sandeep, Narges and Gemma.

I am grateful to have wonderful friends outside work as well, without whom my life in Uppsala wouldn’t have been the same. I would like to thank Maruthi garu & Chandana, Varun & Pratyusha, Vivek garu & Sri Lakshmi garu, Mahesh & Saritha, Kalyan garu & Manjari garu, Hari & Saranya, Chandra & Archana, Sankar & Sandhya and Ram Chaitanya for their love and kindness. I would like to thank the whole Telugu community in Uppsala for their hospitality and generosity. Thank you for making me feel like I was not away from home and my family. I would also like to thank my wife’s colleagues for their support and kindness during our difficult times. Thanks to Monica, Maaret, Yvonne, Rama, Jaya, and Jan. It is always a pleasure to meet you with some nice food around.

I would like to express my gratitude to my friends and family in Finland. I am grateful to have a family far away from home that accepted me as one among them. I would like to thank Eija and Martti from the bottom of my heart for all the love and compassion they have shown. I would also like to thank Tuomas, Tuuli, Antto, and Pinja for extending their love and warmth during family gatherings. I would like to thank Tanuj & Shravya and Naveen for their hospitality, movies, memorable trips, games, and discussions. Most importantly, I am grateful to my parents, who have been put through several hardships to provide us as best as they could. Their blessings, love and support have made me what I am today. I would like to thank my brother Sai, who shares my views in several aspects of life. Thank you for everything you have done and for being a good brother. I would like to thank my uncle Vijaya Kumar and aunt Udaya and their family for their love and support during crucial phases of my education. I am forever grateful to Chinnammayi aunty and her family for helping me in my journey to Nordics. Things wouldn’t have been same without you in my life. I am truly grateful to the warmth and love of my extended family, especially my loving grandmother Maha Lakshmi. Finally, I would like to thank my lovely wife Sandhya for bringing vibrance and music to my life which otherwise is mostly silent. Bujji, you are an ocean of love and kindness. Thank you for coming into my life and for supporting me in all aspects of this life-journey.
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Acta Universitatis Upsaliensis

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