Biomimetic materials design towards tough nanocomposites and strain-stiffening hydrogels

Lahja Martikainen

The field of biomimetics aims to borrow nature's well-adapted design strategies, structures and functions to solve material engineering problems. This thesis focuses on two nature-inspired systems: (i) tough and strong nacre-mimetic nanocomposites, and (ii) strain-stiffening biopolymer hydrogels and their application for cell culturing. Both systems consist of nanoscale components. Their mechanical properties and connection to structure are studied. Based on a biomimetic materials design approach, the first part of this thesis illustrates a simple method to control the mechanical properties of layered clay-polymer nanocomposites. The second part presents insights into the fibril network mechanics of agarose hydrogels. Finally, the last publication introduces a reliable agarose-based preclinical model, which can be used as a platform for breast cancer drug development and personalized cancer therapy.
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Lahja Martikainen

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Aalto University
School of Science
The Department of Applied Physics
Molecular Materials
Supervising professor
Professor Olli Ilkala, Aalto University, Finland

Thesis advisor
Associate Professor Nonappa, Tampere University, Finland

Preliminary examiners
Professor Alan Rowan, University of Queensland, Australia
Professor Francois Barthelat, University of Colorado Boulder, Colorado, United States

Opponent
Associate Professor Ali Miserez, Nanyang Technological University, Singapore

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Images: Nacreous layer inside the abalone shell, photographer Valeria Azovskaya

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Abstract

Biological organisms use only a few chemical elements to construct materials, such as proteins, polysaccharides, and minerals, which are efficiently processed into hierarchical structures across length scales. These clever multi-component designs produce materials and structure with remarkably improved mechanical properties and functionalities relative to the original components. The field of biomimetics aims to mimic these well-adapted design strategies, structures and functions to solve material engineering problems.

This thesis focuses on two nature-inspired systems: (i) tough and strong nacre-mimetic nanocomposites, and (ii) strain-stiffening biopolymer hydrogels and their application for cell culturing. Both systems consist of nanoscale components and their mechanical properties and structure are studied.

The design strategy for tough and strong composites is inspired by the nacre, a biomaterial with outstanding mechanical properties. Here the structural organization of nacre is mimicked via self-assembling core-shell structured colloidal platelets, i.e. nanoclay coated with a polymer, via vacuum filtration. In publications I and II, we alter the interactions between the colloidal platelets with DNA-based monophosphates and demonstrate a simple way to modify intermolecular interactions resulting in increased stiffness, strength, and toughness. Like natural nacre, these nanocomposites are sensitive to humidity. In publication III, we study the effect of water in polymer-clay nanocomposites and find that the glass transition temperature of the nanoconfined polymer is lowered due to residual water.

The second part is inspired by typical extracellular matrix-based protein gels, which show strain-stiffening. In publication IV, we show that agarose hydrogels are strain-stiffening, consisting of helically twisted semiflexible fibrillar networks. In publication V, we analyze this strain-stiffening response more closely and simultaneously show, for the first time, that agarose gels also contract when sheared, which is seen as negative normal force and normal stress difference. Our main findings indicate that the mechanical response of agarose networks is enthalpic and that connectivity dictates their strain-stiffening response similarly as in collagen gels. Finally, in publication VI, we present an application for agarose hydrogels as a luminal cell identity and estrogen receptor α+-preserving scaffold for breast cancer tissue explant culture.

Base on a biomimetic materials design approach, the first part of this thesis illustrates a simple method to control the mechanical properties of layered clay-polymer nanocomposites. The second part presents insights into the fibril network mechanics of agarose hydrogels. The final publication introduces a reliable agarose-based preclinical model, which can be used as a platform for breast cancer drug development and personalized cancer therapy.

Keywords Bioinspiration, nacre, agarose, nanoclay, nonlinear viscoelasticity, negative normal stress, subisostatic network

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Pages 172

Tekijä
Lahja Martikainen

Väitöskirjan nimi
Biomimeettinen materiaalisuunnittelut kohti sitkeitä nanokomposiitteja ja myötyjäkykyistviä hydrogeelejä

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Tiivistelmä
Biologisten luonnonmateriaalien, kuten proteiinien, polysakkaridien ja mineraalien, pohjana ovat
vain muutamat alkuaineet. Biologisissa organismeissa ne muodostavat sopivia hierarkkisia
komposiittirakenteita, joiden ominaisuudet ja toiminnat ovat moninkertaisesti paremmat
ysittäisiin komponentteihin nähden. Biomimetiikka pyrkii ottamaan mallia näistä luonnossa
esiintyvistä materiaaleista, rakenteista ja toiminnosta ja soveltaa niitä teknisten ongelmien
ratkaisuissa.

Väitöskirjassani keskityn kahteen luontoa esikuvanaan käyttävään materiaalisovellukseen: (i)
lujat ja sitkeät helmääistä jäljittelevät nanokomposiitit ja (ii) myötyjäkykyiset 3D-
solunkasvatusalastat. Molemmissa sovelluskohteissa muokkasin nanokoon komponenteista
koostuvien materiaalien mekaanisia ominaisuuksia ja tarkastelin loppumateriaalien rakenteen
ja ominaisuuksien välisiä riippuvuuksia.

Simpukan helmääisen lujaa ja sitkeää rakennetta matkittiin kolloidisilla hiutaleilla, joiden sisus
koostui yksittäisiä nanosavihiutaleista ja ulkokuu polymeereista. Kolloidiset hiutaleet
itsejärjestyvät kerroksellisiksi nanokomposiittikalvoiksi. Julkaisuissa I ja II muokkasimme
kolloidistien hiutaleiden vuorovaikutuksia DNA-pohjaisilla vetyisäkäyttöineen monofosfaiteilla, mikä
lisäsi lujuttaa, jäykentyttä ja sitkeyttä. Nanokomposiittikalvoit olivat herkkä niiden kosteudelle. Julkaisussa
III tutkimme kosteuden vaikutusta savipolymerinanokomposiittikalvoihin. Näytämme, että
kosteus lisäsi polymereeraavien dynamiikkaa ja alensi nanokomposiitin lasitransitiolämpötilaa, mikä
läsäsi kalvon sitkeyttä.

Väitöskirjani jälkimääräinen osan materiaalien taustalla ovat myötyjäkykyiset proteiinigeelit, jotka
on valmistettu soluvälineen tai solun sisällä kulkevien proteiineista. Julkaisussa IV näytämme,
 että agarooishydrogeelit ovat myötyjäkykyistviä. Julkaisussa V perehdyhimme
myötyjäkykyistymiseloon ja osoitimme, että agarooishydrogeelit puristuvat kokoon
leikkausjännityksessä. Tulokset viittaavat siihen, että entalpia määrittää agarooiseelien
mekaanista vastetta ja verkkoarkeen koordinaatioluokku määrittelee myötäjäkykyistymisvasteen,
kuten aiemmin on havaittu kollageineeleillä. Lopuksi julkaisussa VI näytämme, että
agarooishydrogeeli soveltuu solunkasvatusalastaksi luminaalisille ER-α+ rintasyövillä.

Biomimeettisen materiaalisuunnittelun tulokseina osoitamme väitöskirjassani tavan kontrolloida
savipolymerinanokomposiittien mekaanisia ominaisuuksia. Näytän myös, kuinka
agarooishydrogeelien kuivuterverkkoen saattaa niiden mekaanista käyttäytymistä. Lopuksi
esittelen agarooisopohjaisen hydrogeelikasvatusalatan, joka sopii myös mallialustaksi
syöpälääkäteutkimukseen ja yksilöllisiin prekliniisiin rintasyöpälääkäteutauksiin.

Avainsanat
Biomimikka, helmääinen, agaroosi, itsejärjestyminen, nanosavi, epälineairinen
viskoelasiusuus, negatiivinen normaalijännitys, verkkoarkeen


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Curiosity to learn something new has driven me to seek challenges and say yes to many opportunities. Therefore, it was quite logical to end up doing doctoral research. Retrospectively, I could say that curiosity may help you begin your doctoral research but finishing the thesis requires resilience or even some masochism. Luckily, I have been able to share this amazing adventure with so many wonderful people. You are too many and I cannot mention everyone in this preface. However, I am very grateful to all you for the help and support that have gotten me this far.

The research presented in this thesis has been carried out mainly in the Molecular Materials group, at the Department of Applied Physics, Aalto University School of Science. I want to thank my supervisor, our group leader, Distinguished Aalto Professor Olli Ikkala. You have special talent to inspire people and bring them together from different backgrounds. I have been privileged to be a part of your multidisciplinary group as well the wider scientific community, Center of Excellence, HYBER. You have enabled fruitful surrounding, where I had the opportunity to learn and grow as a scientist. Thank you for everything. I want to express my gratitude to my thesis advisor Assistant Professor Nonappa. Thank you for your help and always keeping your door/WhatsApp open for discussions. It has been a pleasure to work with you.

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I want to present my deepest gratitude to all my co-authors. The close collaboration with Kia Bertula has been crucial. I sincerely thank you for sharing the excitement and frustration related to gels, science, and life in general. Special thanks also to Paulinna Munne and Juha Klefström, for directing us to study agarose gels more deeply. I thank you for the open exchange of ideas and all the guidance. I am grateful to Matti Turunen, without you the data in the negative normal force paper, would have not been that extensive. I need to acknowledge Professor Andreas Walther’s experimental discoveries and Tuukka Verho’s in-depth toughness research related to nacre-mimetics, which both served the basis for my thesis. Thank you!
For practical help and discussions, I want to thank many present and former colleagues in Nanotalo. Especially the people of the Molecular Materials –group, the Soft Matter and Wetting –group, and the Active Matter –group, you created friendly and safe atmosphere. It has been absolute pleasure to get to know you. I hope that the “MolMat Dinosauria” after work beer can be resumed soon so that I can meet you face-to-face again.

I wish to thank Mirkka Jones for taking her time to proofread the manuscript and keeping the HYBER alive. In addition, I want to thank the other HYBER coordinators Marjo Kettunen and Jukka Hassinen for everything. Thanks, Heidi Henrickson and Valeria Azovskaya at Materials Platform, for arranging and inviting Jess Kelley to keep the writing retreats which were essential for me to grow as a writer. Mika Latikka and Ville Hynninen, thanks for the peer support and the practical tips related to graduation.

I am grateful to Maja Vuckovac and Matti Toivonen for welcoming me into the office number 111. Our conversations saved many days when the actual science was not working out as expected. Johanna Majoinen, Associate Professor Jaana Vapaavuori, and the Supernaiset: Jenni Koskela, Susanna Junnila, and Henna Rosilo –thank you for your professional support as well as your friendship.

Without supportive society and flexible funding, this work would have not been possible. This research has been founded by Academy of Finland, ERA-NET Woodwisdom, Business Finland, and ERC. In addition, personal grants from the Finnish Foundation for Technology Promotion, Jenny and Antti Wihuri Foundation, Walter Ahlström Foundation, and Finnish Concordia Fund, are gratefully acknowledged.

I'm blessed to have friends with whom I've shared the joys and sorrows of life. I need to mention Leena Arjanne, who shared her one room apartment with me, and Hilja Mäkinen, who have been always visiting me wherever I've been.

The past decade has been an emotional rollercoaster. The unconditional help and love from my family and in-laws have been irreplaceable! Thank you, Ilona and Tomi, as well as Pirkko and Hannu, for keeping your door open for us. Äiti ja isi, kiitos rakkaudesta ja siitä, että vain olette. Jaakko you know all the good, bad, and ugly. Thank you for pushing and encouraging me to take the next steps. My home is where you are. I love you.

Although, science has thought me many things, most valuable lessons I have learned from children. Thank you, my precious sons Taisto and Raimo, as well as my goddaughters Noora, Pihla, and Vuokko, for teaching what is the most important in life. This is for you.

Espoo, April 23, 2021,

Lahja Martikainen
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Abbreviations

**AFM**  atomic force microscopy

**BMx**  basal promoting matrices

**CFIP**  central-force isostatic point

**dCMP**  2’-deoxycytidine 5’-monophosphate sodium salt hydrate

**dGMP**  2’-deoxyguanosine 5’-monophosphate sodium salt hydrate

**DNA**  Deoxyribonucleic acid

**DSP**  digital speckle photography

**EA**  elemental analysis

**ECM**  extracellular matrix

**ER\(\alpha\)**  estrogen receptor \(\alpha\)

**LBL**  layer-by-layer

**LMx**  luminal preserving matrices

**LVR**  linear viscoelastic region

**MTM**  montmorillonite

**PDEC**  patient derived explant culture

**PDEC-N**  normal healthy patient derived explant culture

**PDEC-BC**  breast cancer patient derived explant culture

**PDADMAC**  poly(diallylammonium chloride)

**PVA**  poly(vinyl alcohol)

**PVAm**  poly(vinyl amine)
Abbreviations

**PBS** phosphate buffered saline

**p38** stress pathway

**RH** relative humidity

**SAXS** small angle X-ray scattering

**SEM** scanning electron microscopy

**TEM** transmission electron microscopy

**WAXS** wide angle X-ray scattering

**WLC** wormlike chain

**wt%** weight percentage
Symbols

A  area

d  spatial dimension

c  concentration

δ  phase shift

E  elastic modulus

ε  tensile strain

F  force

Fz  normal force

G'  storage modulus

G''  loss modulus

G*  complex modulus, dynamic modulus

G₀  plateau storage modulus obtained from frequency sweep

γ  shear strain

γ_c  shear strain at the onset of stiffening

h  gap size

I  area moment of inertia

K  differential modulus

K_{enthalpic}  enthalpic spring constant

K_{entropic}  entropic spring constant

l  length
Symbols

$l_p$ persistence length
$L_c$ contour length
$L_x$ end-to-end distance
$m$ scaling exponent
$M$ torque, moment
$N$ apparent normal stress difference
$N_1$ 1st normal stress difference
$N_2$ 2nd normal stress difference
$r$ radius of polymer fibril or solid beam
$R$ radius of parallel plate
$\sigma$ shear stress
$\sigma_c$ shear stress at the onset of stiffening
$\sigma_{TS}$ tensile stress
$T$ temperature
$T_g$ glass transition temperature
$T_{gel}$ gelling temperature
$T_m$ melting temperature
$z$ average connectivity, i.e. coordination number
$z_{CFIP}$ average connectivity at central-force isostatic point
$\kappa$ bending modulus of a polymer segment
$\tilde{\kappa}$ fiber rigidity
$\kappa_B$ Boltzmann constant
This thesis consists of an overview and of the following publications which are referred to in the text by their Roman numerals.


Author’s Contribution

Publication I: “Deoxyguanosine Phosphate Mediated Sacrificial Bonds Promote Synergistic Mechanical Properties in Nacre-Mimetic Nanocomposites”

The author designed the material and the experiments, based on the original idea of the O.I. and A.W. The author performed all the experimental work, excluding wide-angle X-ray measurements, cryo-TEM imaging and elemental analysis. J.S. performed cryo-TEM. The author analyzed all the results and wrote the first version of the manuscript. O.I., A.W., L.B. and J.S. provided comments and suggestions for analysis and writing of the manuscript. The author finalized the manuscript based on the comments of the other authors.

Publication II: “Cytidine Functionalization Promotes Synergistic Mechanical Properties in Nacre-Mimetic Nanocomposites”

The author designed the material and the experiments, based on the original idea of the O.I. and A.W. The author performed all the experimental work, excluding cryo-TEM imaging and elemental analysis. The author analyzed all the results and wrote the first version of the manuscript and finalized the manuscript based on the comments of the other authors.

Publication III: “Hydration and dynamic state of nanoconfined polymer layers govern toughness in nacre-mimetic nanocomposites”

The author performed clay/polymer nanocomposite film preparation, did preliminary tensile testing and participated in discussion of the results and contributed completing the final version of the manuscript. T.V. designed the main research idea together with O.I and A.W. T.V. and M.K. performed dynamic mechanical
analysis. T.V. carried out dielectric spectroscopy. T.V. and R.L. did the small-angle X-ray measurements. P.D. performed tensile testing. O.I., A.W., and A.A. provided comments and suggestions for analysis and writing of the manuscript. T.V. analyzed the data and was the main author of the manuscript.

Publication IV: “Strain-stiffening of agarose gels”

The author and K.B. designed the original research idea together with O.I. and N. The author and K.B. analyzed all the results and wrote the first and last versions of the manuscript together. The author performed strain-stiffening analysis, AFM-imaging and persistence length analysis. K.B. performed rheological measurements, SEM imaging and connectivity analysis. N. N. performed the cryo-TEM sample preparation and imaging. S.H. provided help with the rheological experiments. P.M., J.K. and S.H. provided comments for writing of the manuscript. O.I. and N. contributed in discussion of the results and writing of the manuscript. First authorship is divided between K.B. and the author.

Publication V: “Strain-stiffening and negative normal force of agarose hydrogels”

The author and K.B. designed the original research idea and wrote the first version of the manuscript together. M.T. carried out pre-strain experiments and analysis based on the author’s plan. The author performed the rest of the rheological experiments and AFM-imaging. The author did the strain-stiffening and normal force analysis. K.B. performed SEM imaging, connectivity analysis, preliminary rheological measurements. O.I. contributed in discussion of the research idea and the results, as well as in the writing of the manuscript. The author finalized the manuscript based on the comments of the other authors. First authorship is divided between K.B. and the author.

Publication VI: “Compressive Stress-Mediated p38 Activation Regulates ERα+ Phenotype in Breast Cancer”

The author contributed in to the planning of the study design based on the original research idea of P.M., J.K., O.I. and N. The author performed rheological studies, SEM imaging, and related sample preparation together with K.B. The author analyzed the rheological results. The author wrote parts of the manuscript and participated in completing the final version of the manuscript together with the other authors. Study design: P.M.M., L.M., K.B., N., H.A.-H., M.K., C. M., M.R.J., L.N., I.R., A.P., J.R., J.K., O.I.; writing group; P.M.M., L.M., K.B., N., I.R., H.J., J.P., J.P., O.M., J.K., O.I. data analysis: P.M.M., L.M., K.B.,

Language check

My dissertation has been proof-read by Dr Mirkka Jones. I have personally examined and accepted/rejected the each of the suggested edits. This has not affected the scientific content of my dissertation.

Declaration of subcontracting

Elemental analysis in publications I and II was subcontracted from Microanalytisches Labor Pascher, Germany. The service provider implements high quality control for its service. The authors carefully evaluated the data which were in line with results acquired by the authors using other characterization methods.
1. Introduction

Nature has always been a source of inspiration for materials scientists. Biological materials are inherently multi-scale and multi-functional, serving several purposes in living organisms. For example, the tree trunk not only provides mechanical support but also a network for distributing nutrients and water. Bones act as an important reservoir of minerals in addition to their main role as mechanical support. Like the examples above, most natural materials occur as composites combining at least two different materials. While the materials presented may have other functions, in this thesis the main focus is on mechanical properties of structural materials controlled across multiple length scales, from the molecular to the macroscale.

Biomimetics, also known as biomimicry and bio-inspired material research is a field of science, which combines materials science and various fields of biology. Biomimetics borrows and mimics nature’s well-adapted processes, structures, and functions to solve material engineering problems. The materials created in this thesis are inspired by nacre, found in mollusk shells, and the fibrillar networks of the cytoskeleton and extracellular matrix (ECM). Nacre, probably most well known from pearls, is an iridescent layer inside the nacre-forming mollusk shell. Its mechanical properties are extraordinary, combining strength, stiffness, and toughness, providing protection against predators. Fibrous protein networks are widely present in intra- and extracellular spaces, supporting cells and tissues. These networks have unique mechanical properties: they are strain-stiffening, helping to preserve tissue integrity under mechanical stress.

Nature not only provides inspiration for the materials prepared in this thesis, but also a primary source of its biomaterials. Nanoclay, natural montmorillonite (MTM), is the most abundant naturally occurring layered silicate. It serves as the basis for nacre-mimetic nanocomposites. The strain-stiffening hydrogels, in turn, are made of agarose, a polysaccharide extracted from some red algal seaweeds.

Biological systems have a limited number of elements available as primary material constituents, nevertheless they encompass a huge structural diversity. Biological structures frequently comprise building blocks, with relatively poor mechanically properties, such as proteins, polysaccharides or brittle minerals.
Yet the resulting multi-component structures have significantly better properties and functionalities than the original starting components. Over evolutionary time, biological systems have adapted and developed supramolecular processes under ambient conditions, resulting in nearly perfect nanoscale components that self-assemble into hierarchical structural materials.

Usually high-performance materials require energy-intensive preparation. However, in this thesis nacre-mimicking films and strain-stiffening hydrogels are prepared under mild conditions via facile self-assembly processes, representing an effective bottom up approach for organized nanostructures. Self-assembled systems have a specific structure arising from competing repulsive and attractive interactions towards a local energy minimum. The chemical composition, shape and size of the constituents affect what kind of structure is assembled. Biological materials, like nacre, bone and spider silk, are formed via dissipative self-assembly. For example, protein folding is a result of competing hydrophilic/hydrophobic and complementary physical interactions like ionic interactions, hydrogen bonding and hydrophobic effects.

1.1 Outline of the Thesis

This dissertation concentrates on the potential of biomimetic structural materials with impressive mechanical properties. Two physically very different biomimetic applications are presented: layered clay-polymer nanocomposite films and agarose-based hydrogels. The first is inspired by the strong and tough biological nanocomposite nacre, and the latter has its inspiration in the protein-based strain-stiffening hydrogels such as collagen. The connecting thread between these material mimics and their inspirations is that they all consist of nanoscale components, which are self-assembled into hierarchical structures. The final structure gives the materials their interesting properties. The main focus throughout the dissertation is on two interesting mechanical properties: toughness and strain-stiffening. Both are related to preventing mechanical failure in natural structures.

This thesis is structured as follows. Chapters 1-3 introduce the basic concepts and background of the design strategies and methods behind the biomimetic material design described in publications I-VI.

Chapter 1 is an introduction to biomimetics and briefly presents the main materials, clay/polymer nanocomposites and agarose hydrogels, used in the thesis. The main sample preparation processes are presented as is the scientific context of the whole study. Further details of the materials and methods are described in publications.

Chapter 2 will present some biological materials with unique mechanical properties and concentrates on how these natural materials prevent structural failure. Nacre and bone are shown as examples of strong, stiff and tough nanocomposites. The main energy dissipation mechanisms which increase the
toughness of biological nanocomposites are described, with a particular focus on the biopolymer layer and sacrificial bonds. The polymer layer is central to the clay/polymer nanocomposites in publications I-III. Some examples of strain-stiffening hydrogels with a fibrillar network structure are presented. The two main explanations, semiflexible fibrils and low connectivity topology concepts, for strain-stiffening are presented as well. A simple comparison of entropic and enthalpic contributions in fibril mechanics is presented and explanations for negative normal stress are briefly introduced.

As the main focus of the thesis is on mechanical properties, chapter 3 summarizes the main mechanical testing methods used in the thesis. The tensile testing of nanocomposite films and the main mechanical concepts are presented. In relation to the measurement of agarose hydrogels the focus is on the difference between amplitude sweep and pre-strain methods. Importantly, the theoretical basis and challenges related to negative normal force are discussed. Other details of the materials and methods are described in the publications.

Chapter 4 represents the main findings of publications I-VI. The first part of the chapter concentrates on clay/polymer nanocomposites (publications I-III): nanocomposite compositions are presented, and their differences are discussed. The effect of DNA-based monophosphates with hydrogen bonding motifs on clay/polymer nanocomposites is presented and the influence of relative humidity on mechanical properties is seen (publications I and II). The effect of water on the glass transition temperature of clay/polymer nanocomposites is also discussed (publication III). The second part of the chapter concentrates on agarose hydrogels (publications IV-VI): The structural findings of publications IV and V (helical twisting, fibril diameter, persistence length and connectivity) are discussed. The strain-stiffening and negative normal force effects of agarose hydrogels are presented, and the results of publications IV and V are compared. The negative normal force results are also discussed (publication V) as well as the origin of the strain-stiffening in agarose hydrogels linking it to concepts presented in chapter 2 and 3. Lastly the application for agarose hydrogels as a luminal identity preserving scaffold is shown (publication VI).

Finally, Chapter 5 offers final conclusions and an outlook on the future of nacre-mimetic nanocomposites, strain-stiffening hydrogels and biomimetic material design.

1.2 Clay/Polymer Nanocomposites as Nacre-mimics

Nacre-mimetic nanocomposites are layered structures with high reinforcement content. Stiff, strong and tough nacre-mimics are obtained via different techniques. For example, ice-templating [30, 88], and various layer-by-layer (LBL) [120, 93, 94, 59] methods have been typically used. From the mechanical point of view, the best performing nacre-mimetic nanocomposites may be ice-templated alumina, Al₂O₃, composites with a yield strength of 200 MPa and fracture tough-
ness of 30 MPa-m^{(1/2)} [88]. Nonetheless, the ice-templating of the ceramic-metal alloy, Al_{2}O_{3} - Al-Si, composite is an energy-intensive processing method owing to the use of a high vacuum for sublimation of ice and high temperatures for sintering of Al_{2}O_{3}.

Natural clay platelets such as MTM are an attractive reinforcement material, which is stiff and strong due to its two dimensional (2D) single crystal structure. The clay nanoplatelets can be self-assembled into well-aligned layered structures. There are also other appealing properties when using clay, such as fire resistance and gas barrier properties. In addition, natural clays, like the MTM used in this thesis, are abundant and cheap. MTM has a 2:1 layer structure and it belongs in the group of smectites, which have a high cation exchange capacity and swelling properties [85].

Clay-polymer composites have been extensively studied since the 1970’s. For example, the car industry used a small fraction of clay reinforcement in the polymer matrix, providing remarkably improved mechanical properties. However, increasing the clay content to above 10 vol%, corresponding to approximately 20 wt%, resulted in inferior properties. Consequently, the inorganic content remained below 10 vol% for many years until new fabrication and modification methods enhanced the exfoliation of the clay platelets in the polymer matrix. A recent review highlights progress in high nanofiller content nanocomposites, presents graphene oxide as a nanofiller and the fabrication methods in detail [43].

Ten years ago, a method of fabricating high nanoclay content nacre-mimics via self-assembly was introduced [129, 130]. The same method was used in this thesis. The preparation process is presented schematically in Figure 1.1. The key feature of the process is core-shell inorganic sheets, that is, individual polymer coated nanoclay platelets made possible by the complete exfoliation of the MTM platelets in water. These polymer-coated clay platelets form a layered structure during vacuum filtration as a result of the stacking tendency of the 2D-sheets.

The clay used in publications I-III is natural MTM, under the product name Cloisite Na+ from Southern Clay Products/ROCKWOOD Clay Additives GmbH, Moosburg, Germany. MTM is permanently anionic and most of the counter-ions are sodium. It has a cation-exchange capacity of 92.6 meq/100 g and a basal spacing of 1.17 nm. In publications I and II the polymer is Poly(diallyl methylammonium chloride), PDADMAC, purchased from Sigm-Aldrich as a 20 wt% aqueous solution with a weight average molecular weight of 200 000-350 000 g/mol. In publication III, 98% hydrolyzed poly(vinyl alcohol), PVA, with a number average molecular weight of 85 000-124 000 g/mol from Aldrich is used as a polymer phase.

To create even stronger and stiffer nanocomposite films both covalent [129] and ionic [130] crosslinks have been used to interlock core-shell platelets. However, due to their rigid nature the resulting materials do not allow sufficient energy dissipation under deformation, which compromises their toughness. To overcome
Introduction

**Figure 1.1.** Preparation of the self-assembled nacre-mimetic nanocomposites. The exfoliated anionic nanoclay platelets are mixed in a large volume of aqueous solution of either neutral poly(vinyl alcohol), PVA, or charged Poly(diallylamine chloride), PDADMAC. Excess polymer was removed by sequential washing with the help of a centrifuge. The nanocomposite films were prepared via vacuum filtration. In publications I and II, some of the counter-ions of PDADMAC are exchanged with DNA based monophosphates to introduce additional hydrogen donors (D) and acceptors (A) into the structure.

In this problem, in this thesis additional hydrogen bonding molecules were used to explore whether these could promote toughness by acting as sacrificial bonds and allow energy dissipation during deformation. DNA-based monophosphates 2’-deoxyguanosine 5’-monophosphate sodium salt hydrate, dGMP, (publication I) and 2’-deoxycytidine 5’-monophosphate sodium salt hydrate, dCMP, (publication II), purchased from Sigma-Aldrich, were used. In publications I and II, humidity is shown to greatly affect the mechanical properties of the PDADMAC-MTM nanocomposites. This led to the development of publication III, where the effect of humidity on the PVA-MTM nanocomposites was investigated in more detail.

### 1.3 Agarose Hydrogels

Agarose is one of the main components of agar, which is extracted from red seaweeds belonging, e.g. to the family Gelidiaceae [63]. In seaweed, agar is located at the cell wall. Agar macromolecules connect cellulose fibrils similarly to hemicelluloses in the plant cell wall. Agar provides mechanical support and is also believed to provide resistance to chemicals [63].
Agarose macromolecules consist of alternating D-galactose and 3,6-anhydro-L-galactopyranose units linked by $\alpha$-1->3 and $\beta$-1->4 glycosidic bonds (Figure 1.2). Agarose is a linear polysaccharide and is practically neutral. Agarose is mainly responsible for the gelling properties of agar. Both agar and agarose are used as gelling agents in the food industry and in the biomedical field, e.g. in protein purification [103].

![Diagram of agarose structure and gelling process](image)

**Figure 1.2.** Agarose is a linear polysaccharide, which dissolves in aqueous solutions at temperatures above $T_m$. Below $T_m$ agarose random coils start to form single and double helices. Below $T_{gel}$ the helices form a fibrillar network at relatively low concentrations. In article IV we show that the fibrils have a helical twist consisting of several protofibrils.

Agarose dissolves in warm aqueous liquids. At high temperatures, above the melting temperature of the gel, $T_m$, it exists as random coils. When the temperature is lowered enough it starts to form single and double helices [106], which coil together and form fibrils. Finally, below gelling temperature $T_{gel}$, fibrils constitute a network. In publications IV-VI, UltraPure™ Low Melting Point Agarose with $T_m = 65.5$ °C and $T_{gel} <25$ °C from Invitrogen™, (lot: 0000520356) was mainly used.

The formation of the helices is due to the $\alpha$-linkages, which force the macromolecule to curve. The left handed double helix arrangement with 1.4 nm diameter, 1.90 nm pitch and small internal cavity 0.45 nm was first reported by Arnott in 1974 [3]. According to current knowledge, both single and double helices are present [106]. In this thesis we show that the agarose helices continue to coil together and form helically twisted fibrils with a lateral diameter of approximately 10 nm. The fibrils resemble those reported for carrageenans [108].

Agarose gels show higher stiffness compared to gels consisting of helical twisted fibrils, like gelatin [32]. At concentrations above 0.3 mg/mL [52] agarose forms a mechanically percolating network, which retains a large amount of aqueous solution and defines it as a hydrogel. The double helices seem to be the source of strength. It has also been suggested that water molecules
contribute to the agarose network structure, which would partially immobilize water molecules and increase the modulus [12]. However, several studies suggest that large amounts of water are loosely bound to the network [57, 12]. After the gel has formed it starts to slowly release water, and to partially deswell. The release of water is called syneresis and it is an interplay between the elastic energy of the network and osmotic pressure caused by the water [13].

Agarose- and agar-based hydrogels have been studied for over a century. Usually, however, gels with concentrations above 1 wt% (10 mg/mL) have been used. The strain-stiffening effect of hydrogels is typically more pronounced at low concentrations as has been shown with collagen [124, 87]. The use of smooth rheometer geometries easily results in slippage of the gel. This is why the nonlinear effects presented in this thesis have often been missed. In publications IV and V, the nonlinear effects of agarose hydrogels are studied at concentrations of 1.5 - 10 mg/mL and mainly cross-hatched parallel plate geometry is used.

In publication VI, an application for agarose hydrogels as a luminal identity and ER\(\alpha^+\) preserving scaffold material is shown. For these, also higher agarose concentrations are used (mainly between 10 - 70 mg/mL). These stiffer gels do not show a similar strain-stiffening response to that of agarose hydrogels below 10 mg/mL. It is likely that the strain-stiffening effect is also present, to some extent, in gels with agarose concentrations above 10 mg/ml. However, the effect is not as pronounced as at lower concentrations, and other effects, such as slippage or syneresis, might partly or completely mask the strain-stiffening behavior.
2. Lessons from Nature: Preventing Failure

Biological materials, such as collagen fibrils, nacre, bone and spider silk are formed from nanoscale components via self-assembly. There are clear benefits of assembling structures from nanosized elements. Although defect-free one-component materials may have the highest strength and stiffness, they usually lack toughness and flaw tolerance. It is a fundamental challenge to make defect-free single-scale bulk structures. A large enough defect (i.e. a crack or crack like inclusion) in a homogeneous bulk material decreases its strength due to the stress concentration around the defect [62].

Natural materials usually have several hierarchical levels in their structure. For example, red abalone shells have approximately five and bone has around seven hierarchical levels. Moreover, many polysaccharide and protein macromolecules tend to form helices, which form higher hierarchical structures. Adding one hierarchical structural level will reduce variation in mechanical properties, even though complexity will increase [84]. In addition, the helical conformation of protein networks has been reported to enable large dissipative regions around structural flaws protecting against catastrophic failure [1].

The assembly of structures from nanoscale components is a strategy to maintain a uniform quality of properties but also to prevent mechanical failure of load-bearing structures. Adding hierarchical levels to materials consisting of brittle building blocks makes them less sensitive to cracks and increases their toughness [111]. Statistically, in smaller structures there is a smaller probability of having defects larger than the critical size. Materials with defects that are smaller than the critical size do not undergo similar stress concentration and behave basically like defect-free structures. Consequently, small nanoscale structures are less likely to fracture than macroscopic structures and a hierarchical structure comprising nanoscale components is more likely to be defect-free despite assembly errors.

Strain-stiffening fibrillar hydrogels and tough highly reinforced ceramic composites have fundamentally different mechanical properties. However, both of them have several hierarchical levels in their structure from the atomic to the macroscale. Importantly, both these material categories incorporate mechanisms that are believed to be important in protecting the mechanical integrity
Lessons from Nature: Preventing Failure

of biological organisms.

2.1 Tough Biological Nanocomposites

Mechanical load bearing natural materials are usually nanocomposites combining nanosized hard components with a softer phase. For example, spider silk, wood, bone, teeth, nacre, and the exoskeleton of arthropods all consist of a softer biopolymer phase that binds the hard reinforcing nanocomponents together. The nano-prefix usually refers to the fact that at least one of the particle dimensions is less than 100 nm.

Tough materials can dissipate mechanical energy before final failure. If a material is tough, it has mechanisms that can prevent crack propagation effectively. When a tensile test specimen breaks, two new surfaces are created. The area under the stress-strain curve represents the amount of mechanical energy needed to create new surfaces in the structure. High toughness is seen as a large area under the stress-strain curve. If one of the following stiffness, maximum strength or maximum strain is increased, the mechanical energy stored and dissipated by the material increases.

High stiffness and strength are universally desirable for structural materials with a minimum weight. Ceramics have high specific strength and stiffness, however, they are inherently brittle, lacking toughness, which limits their practical use. Biopolymers show ductility. However, they have low specific stiffness and strength, which also lead to low toughness. Usually stiffness and strength can be combined, whereas the challenge is how to combine all three mechanical properties: high stiffness, strength, and elongation. If you increase one of the three mechanical properties the other two, or at least one of them, is typically reduced. Simultaneous increases in stiffness and elongation are an especially formidable challenge. Materials only appear stiff when stress increases faster than strain. Basically, a stiff, strong and tough material should include a high amount of reinforcement, which makes it initially stiff, but it should also include energy dissipation mechanisms, which are activated after a certain stress level. These mechanisms are discussed later in this chapter.

Here bone and nacre are presented as examples of tough, high ceramic content biopolymer nanocomposites. From a simple materials engineering point of view, bone is a fibril-reinforced and nacre is a platelet-reinforced biopolymer nanocomposite. Hard ceramics or minerals are strong under compression but not under tension, whereas softer biopolymers are strong under tension but lack the strength under compression. Clever composite structures allow the synergistic combination of toughness, high strength and stiffness with minimum weight under both compression and tension. The complex structures of bone and nacre have inspired scientists to find ways mimic the mechanisms behind their outstanding mechanical properties.
2.1.1 Bone

Bone is tough nanocomposite of a soft elastic phase of collagen fibers and apatite crystals, which provide stiff mineral reinforcement. The structure and mineral content of bone varies greatly depending on the body site and function. The mineral content of bone is usually 60-70 % of its dry weight. [14].

Compact bone, also known as cortical bone, consists of osteons (Figure 2.1). The outer concentric lamellae layers have a plywood arrangement, where lamellae have different collagen fiber orientations. The fibers have a diameter of 5 $\mu m$ consist of mineralized collagen fibrils with a diameter of 500 nm which are bound together with nonfibrillar collagen. Mineralized collagen fibrils are composites of topocollagen triple helices and hydroxyapatite nanocrystals.[142]

![Figure 2.1. The hierarchical structure of bone.](image)

The mechanical properties of compact bone depend on the loading direction and the mechanical testing method. Typically, compact bone has an elastic modulus of 10-30 GPa and a strength of 100-350 MPa in the longitudinal direction of the osteon's tubular structure [69]. Furthermore, the toughness of bone depends on the principal orientation of collagen fibrils. The maximum fracture toughness of 8-12 kJm$^{-2}$ [92] is achieved when cracks require cutting the osteon’s tubular structure in a transverse direction.

2.1.2 Nacre

Nacre is the iridescent layer within sea shell (Figure 2.2 a). It is a biological nanocomposite produced by nacreous sea shells. Nacre has a brick-and-mortar structure, where aragonite bricks are glued together with a small amount of
Lessons from Nature: Preventing Failure

biopolymer mortar (Figure 2.2 c and d). The primary purpose of the nacreous layer is to create a strong shell together with other layers and to protect the animal from predators. While the outer layer of the shell is strong under compression, the nacreous layer is strong also under tension. The most studied nacreous shells are pearl oysters and abalones. They have some differences in their structure but very similar overall properties [131, 50].

![Image](https://via.placeholder.com/150)

**Figure 2.2.** The hierarchical structure of nacre. a) The nacreous layer is located within the abalone shell. b) Mesostructure shows the thicker polymer layers, mesolayers, repeating after 100-200 μm. c) Approximately 500 nm thick aragonite tiles are stacked in nacre. d) Thin biopolymer layers are located between the aragonite tiles. e) The AFM phase image shows clearly that the aragonite tiles consist of nanosized aragonite grains (50-100 nm) glued together with the biopolymer. b) Adapted from Ref. [67] with permission from Elsevier B.V. 2004, c) adapted from Ref. [83] with permission from Elsevier Ltd. 2007, d) adapted from Ref. [116] with permission from Macmillan Magazines Ltd. 1999, and e) adapted from Ref. [104] with permission from Elsevier Ltd. 2005.

Nacre consists mostly of inorganic platelet-shaped aragonite, which is a polymorph of CaCO3, a brittle ceramic [26]. Platelet shape reinforcement allows more effective packing compared with fibers, leading to approximately 95-98 wt% [44] of aragonite in the final structure. The higher content of reinforcement gives a higher specific stiffness compared with compact bone [133].

The elastic modulus of nacre is approximately 60-80 GPa, its tensile strength is 85 MPa and fracture toughness 1.5 kJm$^{-2}$ in hydrated conditions [8]. It is remarkable that with only roughly 2-5 wt% of biopolymer "gluing" nacre has almost one thousand times the toughness of pure aragonite [53], and an order of magnitude higher toughness than that of alumina [5, 4]. The mechanical performance of nacre is comparable with that of glass- or carbon-fiber-reinforced polymers [5, 4]. In a dry state, the elastic modulus of nacre is around 90 GPa and its tensile strength is 110 MPa, but its toughness is compromised [10, 53]. In nacre, water is needed to plasticize the biopolymer phase and promote toughness.

### 2.1.3 Sacrificial Bonds and Other Energy Dissipation Mechanisms

Several toughening mechanisms are recognized for nacre and bone. The importance of hierarchical levels in these materials was already mentioned at the beginning of this chapter. Basically, the presence of interfaces is important for the dissipation of energy in deformation [11]. The aragonite brick structure together with the polymer phase enable the redistribution of stress and crack
deflection in nacre. The organic phase allows sliding of the aragonite platelets and turns an inherently brittle material into material that deforms effectively plasticly, opposite to elastic.

Frictional sliding of the wavy aragonite reinforcement has been shown to have the largest role in promoting the overall toughness of the whole composite material [9]. Here, the interfaces play a crucial role [11]. The attachment between the matrix and the reinforcing component must be good enough to allow stress-transfer from the weaker polymer phase to the reinforcement, otherwise the structure breaks when the strength of the weaker phase is reached. On the other hand, stress also has to transfer effectively through the weaker phase, which can allow energy dissipation over a larger area.

In Figure 2.3 the fracture of nacre and related mechanisms are presented. Crack deflection makes the crack travel a longer distance between the tiles. A tortuous path (Figure 2.3 a and b) consumes more energy and large enough tiles end up bridging the crack. The loading mode also changes: sufficient stress transfer between the aragonite tiles and biopolymer layer allows the load to spread into a large volume around the crack, known as the process zone, which is seen as whitening of the frontal zone and wake (Figure 2.3 a). In practice, stress whitening is a result of the frictional sliding of tiles, leading to crack bridging in the frontal zone and wake. The cracks scatter light and hence appear white. [7, 11]

![Figure 2.3. The fracture and toughening mechanisms of nacre. a) A schematic presentation of a crack in nacre, b) different zones related to the crack c)-f) show different structural elements related to stress transfer and energy dissipation: c) mineral nanobriges, d) nanoasperities, e) biopolymer stretching and f) platelet interlocking. a) b) adapted from Ref. [7] with permission from American Association for the Advancement of Science 2016, c-e) adapted from Ref. [68] with permission from Elsevier Ltd 2009.

The free sliding of aragonite tiles is limited in nacre, by for example, mineral bridges, nanoasperities and interlocking of wavy aragonite tiles (Figure 2.3 c-f). In addition, biopolymer stretching including sacrificial bonds and hidden lengths has been observed in the organic layer of nacre [116] and bone [122, 37, 25]. The stiffness of the whole structure is not compromised as the unfolding of organic macromolecules initiates only after a certain critical force is achieved [101].
Although we do not expect polymer unfolding, in publication I and II the idea was to add sacrificial hydrogen bonds as a form of monophosphate to the PDADMAC-MTM nanocomposite. Previously, the sliding of polymercoated colloidal nanoclays has been limited by covalent [129] and ionic [130] cross-links, which allow high stiffness and strength of clay/polymer nanocomposites but with reduced ductility. The strength of hydrogen bonds is generally lower than that of ionic or covalent ones. Therefore, more ductility is expected. Moreover, the added small molecules may affect the free volume of polymer, thereby changing the behavior of resulting composite.

For a materials engineering point of view, the deformation behavior of polymers depends on, for example, temperature and strain rate. The presence of small plasticizing molecules also changes the mechanical properties. All these environmental changes affect the characteristic timescales of the structural relaxation processes of polymers. The glass transition temperature, \( T_g \), is related to polymer relaxation processes [119]. Below the \( T_g \), polymers are glassy, brittle materials with fewer dissipative mechanisms, whereas close to and above the \( T_g \) ductility increases, leading to rubbery or viscous behavior. Water molecules can plasticize hydrophilic polymers by increasing the free volume [51, 47]. Segmental friction is reduced and physical interactions are interrupted by water, which accelerates relaxation processes and leads to the shift of \( T_g \) to a lower temperature [51, 47].

In nanocomposites, like nacre and its mimics, the polymer is in a nanoconfined space. Studies of thin polymer films and layers between latex particles have shown that the dynamic processes are altered as a consequence of confinement [102, 112, 41]. The \( T_g \) might shift to lower or higher values, depending on the polymer and the interface [102, 140]. In publication III the \( T_g \) of the PVA-MTM nanocomposite is studied and compared with that of bulk PVA.

### 2.2 Strain-Stiffening Hydrogels

Many hydrogels derived from the components of biological tissues show a unique nonlinear behavior - stiffening when deformed [118]. Strain-stiffening refers to a nonlinear mechanical behavior, in which the network stiffens suddenly after applying a certain network-specific strain or stress. The current understanding is that the cells themselves do not stiffen but the components of the extracellular matrix or the cytoskeleton of cells stiffen [127]. The purpose of these networks is to provide mechanical support to tissues and cells, and to allow the movement of cells [18]. Actin filaments, intermediate fibrils and microtubules are found in the cytoskeleton. Collagen and fibrin are present in the ECM. All five of these have been shown to be strain-stiffening. This is crucial for cell differentiation and tissue integrity [118, 28, 136].

The strain-stiffening is ubiquitous in many biological materials but remain rare in synthetic gels. Polyisocyanopeptide-based hydrogels were the first
synthetic model created to demonstrate the strain-stiffening and its effect on cells [60, 56, 28, 109]. In addition, some synthetic hydrogels based on specific supramolecular structures [40, 132, 123] or triblock copolymers [36] have shown to strain-stiffen.

Gels made of agar [2, 117, 74], hyaluronic acid [19], methylcellulose [79, 78], pectin [110, 128], and alginate [49] are polysaccharide-based hydrogels showing strain-stiffening. However, the strain-stiffening of polysaccharide-based networks has been studied much less than that of protein-based gels. Many strain-stiffening gels have been reported to show also another nonlinear effect, negative normal stress difference, when shear is applied [54]. In publications IV and V, the nonlinear mechanical behavior of the common polysaccharide hydrogel, agarose, is shown and its relationship to the network structure is studied in detail.

The origin of strain-stiffening may arise either from the mechanical properties of individual fibrils or from the mechanical properties of the network. The former relies on the semiflexible nature of fibrils, and the latter purely on a geometrical explanation. Basically, one of the above mechanisms alone or a combination of both explain the final strain-stiffening response shown by a fibrillar network (Figure 2.4). Furthermore, the aligning of fibrils upon shear has shown to have an effect on mechanical properties, as the reorientation of fibrils along the shear direction increases the fraction of fibrils in tensile load [15, 38, 89].

![Diagram](https://via.placeholder.com/150)

**Figure 2.4.** The origin of strain-stiffening: a) the semiflexibility of fibrils and b) the low connectivity of a network. Z represents the connectivity, i.e. the number of connecting fibrils at the nodal point. Adapted with permission from Publication IV. Copyright (2019) American Chemical Society.

Here, readers are referred to review papers [17, 82] and a polymer physics textbook [105] for details of the basic mechanics needed to connect our experimental findings to the broader scheme of semiflexible polymer networks. The review papers also provide an excellent overview of current models for semiflexible polymers and their networks.

### 2.2.1 Semiflexible Fibrils

**Persistence length**

Polymers can be divided into three main categories depending on their chain
conformation in thermal equilibrium: flexible, semiflexible or rigid (Figure 2.5). The conformation depends on how much the thermal energy, $k_B T$, can curl the polymer. The measure of this property is persistence length, $l_p$, which is defined as length where $k_B T$ is enough to significantly bend the fibril:

$$l_p = \frac{\kappa}{k_B T},$$  \hspace{1cm} (2.1)

where $\kappa$ is the bending modulus of the polymer segment.

![Diagram of polymer chain conformation](image)

**Figure 2.5.** Polymer chain conformation depends on the ratio between the contour length, $L_c$ and the persistence length of the chain, $l_p$. $R$ in this figure refers to the end-to-end distance, $L_x$. Reproduced from Ref. [100] with permission from The Royal Society of Chemistry 2014.

Flexible polymer chains, i.e., Gaussian-chains, exist in a random coil conformation. They respond to mechanical stretching like entropic springs. The spring constant (and the modulus) of freely jointed polymeric chains, is directly proportional to the temperature, $T$:

$$F = \frac{k_B T}{n b^2} L_x,$$  \hspace{1cm} (2.2)

where $F$ is the force needed to stretch the chain to a certain end-to-end distance $L_x$ and $n$ is the number of monomers of size $b$. The permanently cross-linked natural rubber or synthetic polymer networks are considered to represent networks consisting of flexible chains.

The wormlike chain (WLC) model (also known as a “Kratky-Porod model”) depicts the mechanical behavior of semiflexible fibrils better than freely jointed polymeric chains. In the WLC model the fibrils are basically modeled as inextensible elastic rods or fibers, which have a finite bending stiffness. This has offered a good basis for many models and simulations of biopolymers (e.g DNA, actin), where the extension of the fibrils contour length might also be allowed.
Experimentally, the $l_p$ of fibrils can be estimated from micrographs [61]. The open-source Easyworm MATLAB software, which is used in publication IV, can be used to define the $l_p$ from images [61]. Easyworm uses three different methods to calculate the $l_p$: the mean square of the end-to-end distance as a function of the contour length, the decay of tangent-tangent correlation as a function of contour length and mean-square deviations to secant midpoint as a function of secant length. All these methods are derived from the WLC model. The first two methods usually provide the best fit for fibrils which are longer than the persistence length and the latter is better for the short stiffer fibrils. Easyworm uses the traced fibrils for random resampling based on bootstrap with replacement (more details in reference [61]).

**Entropic vs. enthalpic contribution**

Semiflexible fibrils and their networks can be modeled several ways. Depending on how the undulations of fibril segments are considered, the models can be divided into two classes: thermal (entropic) and athermal (enthalpic). In thermal models the undulations are maintained through thermal forces [126], while in strictly athermal models, the bent structures result from mechanical (enthalpic) bending. Models that exclude the entropic contribution are much faster to calculate, and therefore, desirable from the practical point of view. Hence, it is good to estimate at which length scales the entropic component of the force-extension begins to play a significant role, and when the enthalpic effect drives the whole mechanical response. Further details on the entropic and enthalpic contributions and models can be found in reviews [17, 82].

Semiflexible fibrils can be modeled as nearly rod-like configurations with a finite bending rigidity to the extent that classical beam theory and Hooke’s law for elastic solids apply [17].

The bending modulus of a beam is

$$
\kappa = EI, \quad (2.3)
$$

where $I$ is the area moment of inertia, which is for a homogeneous cylindrical rod: $I = \frac{\pi r^4}{4}$. Broedersz and MacKintosh [17] derived the following effective entropic spring constant for nearly rod-like semiflexible fibrils

$$
K_{entropic} = \frac{90 \kappa l_p}{l^4}, \quad (2.4)
$$

where $l$ is the length of a fibril or a network segment. Note that $K_{entropic}$ is inversely proportional to the temperature ($l_p = \kappa/(k_B T)$).

Hooke’s law for elastic solids is

$$
F = \frac{AE}{l} \Delta l, \quad (2.5)
$$

which offers a basis for the effective enthalpic spring constant

$$
K_{enthalpic} = \frac{EA}{l}, \quad (2.6)
$$
this is purely mechanical and sometimes also known as the enthalpic response at zero temperature.

These two effective spring constants for stretching can be compared. Basically, it can be assumed that the mechanical response of a fibril will be mainly according to the smaller effective spring constant. Therefore the entropic contribution is expected to have a significant role only if

\[ K_{entropic} < K_{enthalpic} \]  
\[ \frac{90\pi r^4 l_p}{4l^4} < \frac{E\pi r^2}{l} \]  
\[ \frac{45}{2} r^2 l_p < l^3. \]

When the fibril is semiflexible \((l_p = l)\), the effective entropic spring constant is smaller than the enthalpic, if \(l > 5r\).

The differential modulus, \(K = \frac{d\sigma}{d\gamma}\), of actin, neurofilaments and polyisocya-nopeptide gels has been shown to follow universal scaling as a function of stress, \(\sigma\), \(K \propto \sigma^{3/2}\), which is a result of entropic stretching of thermal undulations [17]. Collagen gels seem to violate this universal scaling, with \(K \propto \sigma^{1}\) [65]. Models and simulations propose that for subsistatic athermal networks there is no similar universal scaling [66, 18].

A deviation from universal scaling alone is insufficient to test whether the network is athermal or thermal. Based on simulations, the different scaling of strain at the onset of stiffening as a function of concentration [17, 113, 55], and simultaneous detection of normal stresses [24, 58, 81, 54] with strain-stiffening have been suggested to distinguish thermal and athermal networks. Both are discussed in the next two sub-chapters regarding connectivity and negative normal stress. In publications IV and V, strain at the onset of stiffening and normal stresses are studied in agarose hydrogels.

### 2.2.2 Connectivity

The topological explanation for strain-stiffening origins from the connectivity of the network structure. J. C. Maxwell’s calculation of the equilibrium and stiffness of frames in 1864 [77] showed that a frame, i.e., a simple network, consisting of \(n\) points in space requires \(3n - 6\) connecting lines to appear as stiff. Later it was concluded that the number of so called floppy modes in a network with zero elastic energy is \(n_0 = dn - n_c\), where \(d\) is the spatial dimension, which in 3D is 3, and \(n_c\) is number of connecting lines between points. From this calculation the so called central-force isostatic point (CFIP) is derived as \(z_{CFIP} = 2d\), where \(z_{CFIP}\) is the coordination number or average connectivity of the network (number of fibrils connecting at a junction point) at the isostatic point (Figure 2.4 b) [113, 114].
Lessons from Nature: Preventing Failure

Maxwell’s calculations imply that at a certain connectivity \( z_{CFIP} \) the network (frame) is mechanically stable. Below this threshold the network is considered floppy, as the shape of the frame changes when loaded, which means that the frame is mechanically unstable. In 1978, Calladine extended the equation to \( n_0 - n_{ss} = dn - n_c \), where \( n_{ss} \) represents self-stressed connection lines [21]. Self-stress can stiffen the frame, which reduces the zero modes in the network.

Although Maxwell considered frames that are made from rigid trusses, the main idea seems to hold in athermal hydrogel networks [113]. Sharma together with MacKintosh showed that this point is actually a critical point at which there is mechanical phase transition from the floppy to the stiff stage, and collagen networks seem to follow this prediction experimentally [113, 114]. The transition from the floppy to the stiff stage involves a change from bend-dominated to stretch-dominated mechanical deformation [39, 114].

Entropic networks show a decrease in strain at the onset of stiffening with increasing concentration (\( \gamma_c \propto c^{-2/5} \)) [17]. If the network is considered subisostatic athermal, connectivity will mainly control strain at the onset of stiffening [113, 55, 65]. If connectivity is constant, the strain at which the stiffening sets in is constant. However, strain at the onset of stiffening will decrease if the connectivity of the subisostatic athermal network increases while other network parameters are kept constant.

In the network models, the change of fiber rigidity, \( \tilde{\kappa} = (r/2L_c)^2 \), seems to represent the change of concentration in experiments [66]. Fiber rigidity is directly proportional to the volume fraction of rods, which should be proportional to the concentration change in the experiments [66]. Athermal lattice-based 2D-, lattice-based 3D- and 2D Mikado models have shown that if \( \tilde{\kappa} \) is low enough the network is bend-dominant and onset strain for stiffening is independent of \( \tilde{\kappa} \) (and concentration). This \( \tilde{\kappa} \)-independent regime is fully described by a network of floppy ropelike fibrils [66]. Also, if \( \tilde{\kappa} \) is high enough, the strain at the onset of stiffening is constant, i.e., independent of the both density (connectivity) and fiber rigidity (concentration) [66].

### 2.2.3 Negative Normal Stress

If a rod is rotated in a Newtonian liquid, the liquid tends to escape to the walls of the cup. If a liquid consisting of macromolecules is stirred it may climb the rod as a consequence of normal stresses. This rod-climbing effect, also known as the Weissenberg effect [134], is a result of the elasticity of the liquid, which causes a positive normal force. If this kind of liquid is sheared between rotating parallel plate geometry, the sample tends to push the plates apart. Moreover, if an elastic solid rod is twisted, hoop stress creates a positive normal force on the rod, which results in a reduced radius and extended length of the rod [98].

In contrast, many strain-stiffening hydrogels show negative normal stress when sheared. This effect was represented for the first time in detail by Janmey et al. [54]. There, it was connected to the semiflexible nature of the fibrils, which
have a nonlinear force-extension curve. They argued that the fibrils appear softer in compression than under tension. In shear deformation, approximately half of the fibrils are stretched while others are compressed. However, the stretched fibrils will overcompensate, leading to a situation where the sample tries to pull the rheometer plates closer to each other. Similar conclusions have been suggested by others [23].

Thermal networks made from extensible filaments are expected to have a transition from entropic to enthalpic stretching. However, this transformation is expected to happen later than in the athermal networks composed of rigid rods. Kang et al. [58] and Conti et al. [24], showed that athermal networks can have negative normal stress that exceeds the magnitude of the shear stress. A completely thermal model can predict only negative normal stress equal to shear stress [54], which is why the overshoot of $|N/\sigma|$ can hint at athermal behavior.

The above-mentioned models neglect the effect of water on the network. In more recent studies, the effect of water movement has been pointed out and a phenomenological two-component, so called poroelastic, model has been introduced [29]. The effect is strongly time dependent. If the frequency of the oscillation shear is too high the fluid cannot move with respect to the network. The time constant depends on the polymer but also the density of the network [29, 125]. Similar fluid redistribution with respect to the network has been reported in models based on free energy calculations as well [137, 138].
3. Mechanical Testing

This chapter presents some basic mechanical terms, which are needed to understand the results. Also some essential details related to equipment or methods are explained. More detailed descriptions of the materials and methods are in the publications.

3.1 Tensile Testing of the Clay/Polymer Nanocomposites

Tensile testing is one of the simplest way to determinate the mechanical properties of solid specimens. In tensile testing the specimen is extended at a constant rate until it breaks completely. The force is measured with a load cell and in the simplest set-ups the extension is measured with an extensometer. In this thesis, we are interested in tensile strength, strain, Young’s modulus, and toughness, which are obtained from stress-strain curves. Here stress, $\sigma_{TS}$, and strain, $\epsilon$, refer to engineering stress and engineering strain (in literature sometimes called as nominal stress and nominal strain), which are defined as follows:

$$
\sigma_{TS} = \frac{F}{A}, \tag{3.1}
$$

$$
\epsilon = \frac{l - l_0}{l_0}, \tag{3.2}
$$

where $F$ is the tensile force measured by the load cell, $A$ is the initial (nominal) cross-sectional area of the specimen, $l_0$ is the initial gauge length, $l$ is the elongated gauge length, and $l - l_0$ is elongation.

Figure 3.1 shows a typical stress-strain curve and the basic mechanical variables obtained from a single measurement of the dGMP-PDAMAC-MTM nanocomposite. The tensile strength is the maximum or ultimate tensile stress value reached in the stress-strain curve. In the case of clay/polymer nanocomposites this is also the strength at break. Young’s modulus, also known as the tensile modulus or elastic modulus, is defined from the initial slope of the
stress-strain curve, where the specimen is almost linear elastic. Stress-strain curves sometimes show a so called toe region, which is an artifact caused by the uptake of slack in the specimen. Those artifacts were compensated according to the ASTM standard D882-10 before further analysis.

The nacre-mimetic films in this thesis are thin (approximately 100 \( \mu m \)), therefore the area under the stress-strain curve is used as a measure of their toughness known either as work-to-failure (I and II) or the modulus of toughness (III). The unit of work-to-failure is energy/volume, which measures the ability of the material to absorb energy until final failure. In contrast, for thicker samples there are more sophisticated ways of studying toughness. Fracture toughness measurements have been demonstrated for laminated PVA-MTM bulk nanocomposites [86].

![Typical stress-strain curve of dGMP-PDADMAC-MTM specimen at RH 50 % showing the how the tensile strength, Young's modulus, maximum strain and work-to-failure are defined.](image)

Extensometers do not take into account possible slippage of the specimen, which might result in inaccurate measurements especially at small strains. The dry PDADMAC-MTM based films are inherently brittle, having low strain values at break (0.5 – 2\%). To measure elongation accurately, strain is measured optically in publications I and II using a Digital Speckle Photography (DSP) system. The DSP system records digital images during tensile tests and the software recognizes surface speckle patterns and uses cross-correlation to define the representative strain at each pixel. The average strain in the tensile direction is calculated from the whole visible surface area, and is used to define strain at that measurement point. In publication III the PVA-MTM specimens are more ductile, showing larger strains especially in humid conditions, although the tensile tester's extensometer reading is used.
3.2 Rheology of Agarose Hydrogels

The mechanical properties of gels are usually measured with a rheometer. Here, oscillatory shear rheology is used, where an oscillating shear stress or shear strain signal is applied and a response signal (shear strain or shear stress) is measured. In principle, stress-controlled rheometers apply torque and angular displacement is measured and vice versa, in strain-controlled rheometers. Nowadays, sophisticated software allow very accurate control of both variables. Here, we usually controlled strain to avoid creep.

For parallel plate geometry, shear stress and shear strain are defined as follows:

\[ \sigma = \frac{2}{\pi R^3} M, \]  
\[ \gamma = \frac{R}{h} \theta, \]

where \( R \) is the radius of the plate, \( M \) is torque (also known as moment), \( h \) is the gap size, and \( \theta \) is deflection angle (angular displacement) of the shaft. Some of the parameters are visualized in Figure 3.2. The shear stress above is actually approximation and is exactly valid only for Newtonian fluids.

Figure 3.2 b) shows an oscillation cycle of agarose gel. At a high enough frequency the phase shift between the signals, \( \delta \), is visible. From the measured amplitudes and their phase difference, the storage modulus, \( G' \), and the loss modulus, \( G'' \), are defined:
\[ G' = \frac{\sigma_0}{\gamma_0} \cos(\delta), \quad (3.5) \]

\[ G'' = \frac{\sigma_0}{\gamma_0} \sin(\delta) \quad (3.6) \]

The storage modulus describes the ability of a material to store energy and the loss modulus the capability to dissipate energy. The former is the in-phase component and the latter is the out-of-phase component of the complex modulus, \( G^* \), also known as the dynamic modulus. \( |G^*| \) represents the overall resistance of the gel to deformation and it is calculated from stress and strain amplitudes.

\[ G^* = G' + G''i, \quad (3.7) \]

\[ |G^*| = \frac{\sigma_0}{\gamma_0}. \quad (3.8) \]

### 3.2.1 Strain-Stiffening

Strain-stiffening can be studied via shear rheology with at least three different methods. Amplitude sweeps, pre-strain or pre-stress methods and strain ramps have been used in literature [16]. Here, strain amplitude sweep and pre-strain methods are used. In Figure 3.3 the main differences between the methods are presented.

![Figure 3.3. Difference between the pre-strain protocol and amplitude sweep. a) in the pre-strain protocol, static strain is applied and small amplitude oscillations are used b) in the amplitude sweep, the amplitude of the oscillation is increased after each measurement point. Reprinted with permission from Publication V. Copyright (2020) American Chemical Society.](image)

In dynamic amplitude sweeps, a constant frequency is maintained and the amplitude of a sine wave is increased after each cycle. In practice, the rheometer
applies several oscillations at each amplitude and after reaching a certain level it will either record one oscillation or the average of several oscillations. As the frequency is kept constant, the strain rate changes as the amplitude changes. For viscoelastic materials this might be a problem. Furthermore, several high amplitude cycles may promote slipping of the specimen. Hence, we consider the pre-strain protocol to be more reliable. However, the same parameters can be obtained with both methods as is presented in publications IV and V.

$G'$, $G''$ and $G^*$ are valid only within the linear viscoelastic region, LVR, where the oscillation signals are sinusoidal. Thus differential modulus $K$ is used:

$$K = \frac{\delta \sigma}{\delta \gamma}. \quad (3.9)$$

For amplitude sweeps, $K$ is basically the same as $|G^*|$. Here we obtain it from the shear stress, $\sigma$, vs. shear strain, $\gamma$, data as a first derivative by using MATLAB functions spline and fnder (See Figure 3.4 a). For pre-strain measurements, $K$ is not just the ratio between shear stress and shear strain amplitudes as for amplitude sweep data. Instead, it is obtained from the raw stress and strain signal data, which is recorded with the sine-wave-generator of the RheoCompass software.

![Figure 3.4](image)

Figure 3.4. a) Defining the differential modulus, $K$ from amplitude sweep data. b) Defining the stress at onset of stiffening $\sigma_c$ (in publication IV i.e. critical stress) from the $K$ vs. $\sigma$ curve. LVR represents the linear viscoelastic region, where basic rheological functions apply.

Typically, the interesting strain-stiffening parameters are strain at the onset of stiffening, $\gamma_c$, stress at the onset of stiffening $\sigma_c$, and the scaling exponent, $m$ (See Figure 3.4 b). In addition, the scaling relations of these parameters as a function of a concentration have received a lot of interest, as these may reveal the enthalpic or entropic nature of the network.

### 3.2.2 Negative Normal Stress

During rheological measurements, changes in normal force can be detected with a z-direction force sensor coupled with the upper plate. The z-direction is the normal direction of the sample (See Figure 3.2). Typically, all shear rheometers have this normal force sensor as it is used when sample is loaded and compressed.
between the upper and lower plates. This force is used for calculating the normal stress difference. The normal stress differences are used as then the isotropic pressure is canceled from the equations. The 1\textsuperscript{st} normal stress difference, $N_1$, and the 2\textsuperscript{nd} normal stress difference, $N_2$, are defined as follows:

$$N_1 = \sigma_{\theta \theta} - \sigma_{zz} \quad (3.10)$$

$$N_2 = \sigma_{zz} - \sigma_{rr} \quad (3.11)$$

For a Newtonian liquid, $N_1$ and $N_2$ are both zero. For liquids that show elasticity or elastic solids $N_1$ is positive and larger than $N_2$, which is negative. $N_1$ can be defined only for cone and plate geometry. The normal stress difference, $N$, for parallel plate geometry is actually the difference between $N_1$ and $N_2$. Macosko has shown the following approximation for $N$ [73]:

$$N = N_1 - N_2 = \frac{4F_z}{\pi R^2}. \quad (3.12)$$

In this thesis, we refer to this as the apparent normal stress difference because its is true only for incompressible materials and hydrogels seem to violate this principle.
4. Results and Discussion

4.1 Clay/Polymer Nanocomposites, Publications I-III

In publications I-III, we created intercalated clay-polymer nanocomposites. The key elements are the polymer-coated colloidal clay platelets, which form nacre-mimetic films via self-assembly. The nanoclays were covered either with cationic PDADMAC (I and II) or with neutral PVA (III) to form core-shell platelets. PDADMAC has been extensively studied in charged poly(4-styrene sulfonic acid sodium salt), PSS-PDADMAC, assemblies, for which the driving force for the complexation is the gain of translational entropy[115]. Here, the gain of entropy originating from the release of counterions and water molecules at the MTM surface is again the main driving force.

The individually polymer-coated nanoclay platelets were confirmed by AFM, which revealed approximately an 1.2 nm thickness increase. The stacking tendency of the clay platelets resulted in aligned lamellar self-assemblies of alternating MTM and polymer layers (PDADMAC or PVA), which were visualized by high-resolution transmission electron microscopy (TEM). Moreover, scanning electron microscopy (SEM) showed nicely layered structures of stacked platelets.

The PDADMAC-coated colloidal nanoclay platelets were further decorated either with hydrogen bonding guanosine groups (dGMP), publication I, or cytidine groups (dCMP), publication II. Half of the films were prepared with dGMP or dCMP, and half acted as reference films, without the monophosphates. The addition of the monophosphates did not lead to any significant changes at the microstructural level detected with SEM or cryo-TEM.

X-ray scattering and elemental analysis (EA) revealed differences in composition and nanostructure between different clay/polymer films (Table 4.1). All the films have a high content of inorganic reinforcement (at least 70 wt%) and only a small amount of the organic polymer. The PVA-MTM nanocomposites have almost one third more polymer in their structure than PDADMAC-MTM. This is a result of hydrogen bonding in PVA, which allows more polymer to be bound together, whereas the charges of cationic PDADMAC limit the amount
Table 4.1. The composition of the nanocomposites based on elemental analysis results. Basal spacings are defined from the SAXS and WAXS results.

<table>
<thead>
<tr>
<th>Nanocomposite</th>
<th>Polymer fraction (wt%)</th>
<th>Clay fraction (wt%)</th>
<th>Monophosphate fraction (wt%)</th>
<th>Basal spacing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA-MTM</td>
<td>30</td>
<td>70</td>
<td>-</td>
<td>Dry: 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RH 50%: 2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RH 75%: 2.75</td>
</tr>
<tr>
<td>PDADMAC-MTM</td>
<td>23 ± 2</td>
<td>77 ± 2</td>
<td>-</td>
<td>Dry: 1.85</td>
</tr>
<tr>
<td>dCMP-PDADMAC-MTM</td>
<td>18 ± 2</td>
<td>74 ± 2</td>
<td>8 ± 1</td>
<td>Dry: 2.14</td>
</tr>
<tr>
<td>dGMP-PDADMAC-MTM</td>
<td>20.9 ± 1.2</td>
<td>72.0 ± 1.2</td>
<td>7.1 ± 0.7</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

of PDADMAC in the shell. The same is seen also in the basal spacing based on small- and wide-angle x-ray scattering (SAXS and WAXS) results. In PVA-MTM the structural layer is almost 1 nm thicker than in PDADMAC-MTM. WAXS results also revealed that the monophosphates increase the spacing within PDADMAC-MTM, which further confirms the presence of monophosphates in the overall structure.

Figure 4.1 shows stress-strain curves obtained from tensile testing at different relative humidities, RH. All the nanocomposite films show similar trends of decreasing Young’s modulus and tensile strength with increasing humidity. In most cases, the strain increases simultaneously.

Figure 4.1. Stress-strain curves of the nacre-mimetic nanocomposites a) PDADMAC-MTM, b) dGMP-PDADMAC-MTM, c) dCMP-PDADMAC-MTM and d) PVA-MTM, at different relative humidities indicated in the subfigures. d) Adapted with permission from Publication III. Copyright (2013) WILEY-VCH Verlag GmbH & Co.
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Typically, water increases the free volume of the polymer, which lowers the $T_g$ and results in lower strength and stiffness and higher plastic deformation, as discussed in sub-chapter 2.1.3. The $T_g$ of bulk PDADMAC at 10 RH% is approximately 60 °C, and at 50-60 RH% the $T_g$ is close to room temperature [72]. However, a single glass transition temperature is a bit misleading, especially in the case of thin polymer layers. It has been shown that the parts of the polymer connected to the surface, middle film, and free surface have clearly different mobilities and locally different $T_g$ [140], which means that polymers in nanoconfined spaces may behave differently compared with the bulk. The $T_g$ of bulk PVA and the PVA-MTM nanocomposite are compared in publication III.

PDADMAC-MTM without monophosphates differs from the other films as the maximum strain is almost the same despite changes in RH. All the other films show increasing strain with increasing relative humidity. PDADMAC creates ion-dipole connections with water, which are weaker than the hydrogen bonds. [139] Water reduces the attraction between oppositely charged intrinsic ion pairs and facilitates the relaxation of charged assemblies. [141] In our case it seems that water molecules facilitate the movement of the hydrogen bonding motifs without completely breaking the interaction, and disrupt bonding based on solely ionic interactions.

4.1.1 Effect of DNA-based Monophosphates, Publications I and II

The EA confirmed the presence of monophosphates in the structure. Based on the WAXS results, we infer that the dGMP molecules are highly tilted between the core-shell structures. Even though WAXS data is not available for dCMP-PDADMAC-MTM, the results are expected to be similar due to the structural similarity between dCMP and dGMP.

Figure 4.2 illustrates the experimentally collected mechanical testing data for PDADMAC-MTM without and with monophosphates at different relative humidities. First, we observed that in a dry state, the tensile strength of the dCMP- and dGMP-treated samples is higher compared with the original PDADMAC-MTM without monophosphates. This indicates that the monophosphates have an active role in the structure, as strength increases although the fraction of the low density phase is increased. A similar effect is seen also at higher relative humidities. However, at high humidity the difference between the tensile strength and Young's modulus almost disappears. Finally, the maximum strain at failure increases only with monophosphate-containing films, which leads to higher work-to-failure.

At 50 % relative humidity, tensile strength, Young's modulus and maximum strain increased 41 %, 33 % and 5 %, respectively, as a result of dGMP treatment, leading to a 48 % higher work-to-failure, which indicates higher toughness. Similarly, at 60 % relative humidity the dCMP treatment increased tensile strength, and maximum strain 34 % and 69 %, respectively, while Young's modulus remained at the same level leading to a 123 % higher work-to-failure.
Results and Discussion

![Graphs showing mechanical properties](image)

**Figure 4.2.** The effect of monophosphates (dCMP and dGMP) on the mechanical properties of the PDADMAC-MTM nacre-mimetic nanocomposite: a) Tensile strength, b) Maximum strain at failure, c) Young's modulus and d) Work to failure at different relative humidities, RH % presented as averages with standard deviations.

We suggest that the monophosphates introduce additional sacrificial bonds to the structure and act as a viscous dissipating phase.

### 4.1.2 Hydration and Dynamics of the Clay/Polymer Nanocomposites, Publication III

In publication III, the mechanical properties of the PVA-MTM nanocomposite films were studied at even higher humidities than in publications I and II. In addition, to tensile testing, dynamic mechanical analysis, DMA, was used as a direct tool to measure polymer dynamics. In Figure 4.3 a) the PVA bulk is compared with the PVA-MTM nanocomposite and a clear switch in the loss modulus peak to a lower temperature is seen. Hydration decreases the temperature at which the loss modulus peak is seen even further.

The peak seen in nanocomposites is broader (Figure 4.3 a)). This is most likely due to the fact that there are loosely bound PVA chains as well almost completely restricted, tightly bound, PVA chains between the MTM clay sheets. The presence of polymer, with a different mobility, broadens the peaks, as has been observed in semicrystalline polymers and nanocomposites [71, 35]. The elastic modulus (Figure 4.3 b)) seems to exhibit similar behavior, however the slopes in nanocomposites are less clear than in pure PVA.
The temperature at which the peak of the loss modulus and drop of the elastic modulus are seen is defined as the $T_g$. In Figure 4.3 c) $T_g$ is plotted as a function of relative humidity. $T_g$ falls below room temperature at relative humidities above c. 50 %. Typically, nanometer scale confinement of a polymer between strongly adhesive interfaces will cause a shift in $T_g$ towards higher temperatures [102]. Here, the decrease in $T_g$ is connected to the residual water between the MTM layers.

Tensile testing showed that at room temperature hydration exceeding 50 % RH led to a dramatic change in toughness, which was seen as a larger work-to-failure (also called as modulus of toughness). In highly hydrated conditions stable crack propagation was observed. In addition, stress whitening was observed, which indicates the presence of a macroscopic process zone ahead of the propagating crack. This parallels findings for DMA, and means that as the dynamics of the polymer phase change from brittle to ductile, dissipative sliding of the MTM platelets in the nanocomposite becomes possible.

It is extremely difficult to achieve high ductility and toughness without compromising stiffness. If extreme stiffness is not required, adding more polymer into the clay/polymer nanocomposite may improve the toughness further. For example, a lower MTM content (50 wt% of MTM) results slightly lower Young’s modulus, while ductility and toughness increase, in the PVAm/PVA MTM nanocomposite [34].

### 4.2 Agarose Hydrogels, IV-VI

In Publications IV-VI, agarose hydrogels either made in PBS (IV and VI) or pure water (V) were studied. In publications IV and V, we carried out a systematic rheological and electron microscopy analysis of the agarose hydrogel network. A careful analysis of the data allowed us to correlate the network structure, the rheological properties, and finally the enthalpic and entropic deformation of the semiflexible fibrils. In publication VI, the application of agarose hydrogels as an
Results and Discussion

estrogen receptor positive (ER+) and luminal cell identity preserving scaffold is studied.

4.2.1 Structure of the Network

In chapter 1 the basics of agarose gelling were presented. In Figure 4.4 micrographs present the fibrillar network structure found in agarose hydrogels prepared in H$_2$O. The important parameters of networks are mesh size, fibril diameter, persistence length, and connectivity. The key question is how to these parameters respond to changes in agarose concentration? Qualitatively, the network structures seem self-similar, having fibril-poor and fibril-rich areas, and a mesh size ranging from 10 nm to the micrometer scale, almost independently of the concentration.

![Figure 4.4](image)

**Figure 4.4.** a) A cryo-TEM micrograph of 0.83 mg/mL agarose hydrogel b) An AFM height image of 5.1 mg/mL agarose hydrogel, inset, shows a phase image, which clearly reveals the helical nature of the fibrils. c) SEM micrograph of 1.5 mg/mL agarose hydrogel. d) The connectivity, $z$, of the agarose fibril network appears to remain constant over the concentration range. Adapted with permission from Publication IV. Copyright (2019) American Chemical Society.

In publications IV and V, the agarose fibril diameter is estimated from micrographs obtained by AFM, SEM and cryo-TEM imaging (Figure 4.4). All the microscopic techniques gave similar overall results (diameters between 6.3 – 19.7 nm), albeit small differences between the results are observed. The SEM micrographs show that the diameter decreases slightly with increasing agarose concentration. In contrast, cryo-TEM did not confirm this increase (IV). Cryo-TEM imaging does not require any coating or drying of the gel specimen, hence, the diameter of 9.9 ± 0.5 nm obtained by the cryo-TEM technique is the most reliable diameter of the agarose fibrils. Although freeze-drying from liquid propane (IV) and critical-point drying (V) were used to prevent drying artifacts prior to SEM imaging, it is likely that the thin fibrils are more easily drawn together in a fibril sparse network due to the capillary forces resulting in a
larger fibril diameter in low concentration gels.

Helical fibrils have often very well-defined finite diameters as a result of chirality transfer leading to elastic costs which frustrate the lateral assembly [46]. In publication IV, we show that agarose fibrils show a clear helical twist (Figure 4.4 b). Previous illustrations of agarose networks have shown lateral stacking of helices following the schematic image of Arnott [3]. Our AFM micrographs show, first time, that usually two agarose pre-fibrils will coil together forming a larger fibril. A similar behavior has been reported in the literature for another polysaccharide, κ-carrageenan [108].

The connectivity of the agarose hydrogels was estimated from SEM images (IV and V). In spite of the difficulty in distinguishing the junction points, average connectivity appeared to stay between 3 and 4 independently of the agarose concentration (Figure 4.4 d)). This is first indication that the agarose network could be considered as a subisostatic network, with connectivity less than 6 [113].

Despite the fact that the structural analysis of hydrogels by microscopy techniques is challenging and that sample preparation may introduce artifacts, the diameter and mesh size in our experiments are consistent with the earlier findings. In the literature, the radii of agarose fibrils and the mesh size of agarose networks have been characterized by multiple techniques including light scattering, electrophoresis, NMR spectroscopy, and turbidity measurements [6]. The agarose type and concentration affect the gelation kinetics leading to various mesh sizes at concentrations below and near 10 mg/mL, while diameter is independent of concentration [6, 107]. Moreover, the observed helicity supports the finding of a constant, concentration independent diameter of agarose fibrils [46].

Semiflexible fibrils are defined by their persistence length as shown in chapter 2. Here, the \( l_p \) was obtained from AFM micrographs using the Easyworm software [61] (Figure 4.5). In publication IV, altogether 50 fibrils were used in the analysis with contour lengths, \( l_c \), 600 ± 300 nm (Figure 4.5 c)). Here the mean square end-to-end distance against the contour length gave the best fit (Figure 4.5a). The resulting \( l_p \) is 1300 ± 500 nm. The other two methods (the decay of tangent-tangent correlation as a function of \( l_c \) and mean-square deviations to secant midpoint as a function of secant length) gave similar results, 1000 ± 400 nm and 1000 ± 500 nm, respectively, when the fit was done only for lengths less than 600 nm or 400 nm.

The main problem with defining persistence lengths from images is that the fibrils should be close to their 2D equilibrium structure. Basically, this means that the fibrils should be individual and that both of the polymer ends should be visible. Agarose forms a network while gelling. As a result of the gelling kinetics there will be fibril-sparse and fibril-dense regions [6]. Our sample preparation technique led to areas with only a few individual fibrils. However, a total of almost 50 individual fibrils were possible to detect with AFM (Figure 4.5 b)).

The kurtosis analysis and the fractal exponent analysis hinted that the fibrils are not fully in thermal equilibrium. Therefore, surface parameter 1.5 ± 0.5
Results and Discussion

Figure 4.5. The persistence length defined from AFM micrographs via Easyworm software[61]. a) the square end-to-end distance, $R^2$, plotted against the contour length, $l$, the best nonequilibrium fit to the data gave persistence length, $l_p$, of $1278 \pm 449$ nm. b) An example of a AFM image of the traced fibrils used for defining the $l_p$. The trajectories are presented next to the individual fibrils. c) All the trajectories of the fibrils used, totally 50 individual agarose fibrils with average contour length of $617 \pm 201$ nm were used in the analysis.

was used for fitting, which is for a nonequilibrium fit. At thermal equilibrium, the fractal exponent should be 0.75 for $l_c > l_p$. Here the slope is 0.9. Kurtosis should be close to 3, if the fibrils are close to their equilibrium structure. Here the kurtosis was between 3-5 and increased around 800 nm.

Agarose fibrils are semiflexible as $l_p$ and $l_c$ are at the same length scale and the fibrils are present in slightly bent conformations. Equation 2.9 can be used to estimate whether the mechanical deformation of the fibril is based on entropic or enthalpic stretching. If $r = 5$ nm, $l_p = 1300 \pm 500$ nm are substituted, the mechanical response will be entropic only if the mesh size is larger than $75 - 100$ nm. The mesh size of the agarose gels ranges from 10 nm to the micrometer scale. Consequently, the fibrils in the dense regions of the gel are behaving like stiff rods and in the sparse regions of the gel as semiflexible fibrils.

4.2.2 Strain-Stiffening

The strain-stiffening of agarose hydrogels prepared in PBS and H$_2$O was studied with two methods, amplitude strain sweep and pre-strain protocol, which were presented in the chapter 3. Similar results were obtained with both methods. However, the strain amplitude sweep was found to be more sensitive to slipping, which might be the cause of smaller scaling exponents seen for the stiffening region (Figure 4.6 a vs. b).

Although the deviation from universal scaling $K \propto \sigma^{3/2}$ within the stiffening region suggests that the stiffening of the agarose networks does not arise simply from the entropic stretching of the fibrils, it is insufficient to the distinguish enthalpic or entropic nature of the network.

The scaling relations with concentration are seen in Figure 4.7. The plateau storage modulus, $G_0$, (Figure 4.7a) and the stress at onset of strain-stiffening, $\sigma_c$, (Figure 4.7b) scale similarly with concentration. We get similar scaling exponents independent of whether strain amplitude sweeps or the pre-strain
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Figure 4.6. Strain-stiffening of the agarose hydrogels, shown as differential modulus, $K$, as a function of shear stress, $\sigma$. a) Data obtained from individual amplitude strain sweeps of agarose hydrogels in PBS with six different concentrations at 20 °C and four different concentrations at 37 °C. The black numbers represent the powerlaw of stiffening region. b) Data obtained from pre-strain protocols of agarose hydrogels in $H_2O$ and shown as a average of 4-6 measurements with 95 % confidence interval. Note that the confidence intervals are smaller that the dots in concentrations above 2.5 mg/mL. Red numbers represents the average fit of stiffening region with 95 % confidence interval. a) Adapted with permission from Publication IV. Copyright (2019) American Chemical Society. b) Adapted with permission from Publication V. Copyright (2020) American Chemical Society.

Protocol are used. Small differences in the $\sigma_c$ are seen at temperatures of 20 °C vs 37 °C, but this difference in the scaling exponents falls within the 95 % confidence interval. In Figure 4.7b) another interesting aspect is that stiffening sets in at lower stress levels when the pre-strain protocol is used, which might be a consequence of the fact that the static load that is applied has already pushed the gel into the stiffening region while in amplitude sweeps the stiffening happens slightly later.

The onset strain of strain-stiffening, $\gamma_c$, seems to be nearly independent of concentration (Figure 4.8). Naturally, at the edge of the linear viscoelastic region and when the gels are mostly elastic (small phase angle) the basic Hooke’s law can be applied. As a consequence, if $\gamma_c$ is kept constant and only stress increases, stiffness must increase linearly with stress in the linear elastic area. In publication IV, a slight increase in $\gamma_c$ as a function of concentration is seen, however in publication V we see that the $\gamma_c$ is almost independent and only a very slight decrease is observed. This suggests that the mechanical response of the agarose hydrogel network is dictated mainly by connectivity, similar to that of collagen [113, 114].

4.2.3 Negative Normal Stress

The simultaneous detection of normal stress and strain-stiffening response has been suggested to be a method to distinguish whether the stiffening response
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amplitude sweep 20 °C
amplitude sweep 37 °C
pre-strain 20 °C

\( c_{3.3 \pm 0.3} \)
\( c_{3.2 \pm 0.3} \)
\( c_{3.1 \pm 0.3} \)
\( c_{3.4 \pm 0.4} \)
\( c_{3.1 \pm 0.5} \)
\( c_{2.6 \pm 0.4} \)

\((mg/mL)\)

\( \sigma_0 (Pa) \)

\( 10^{-2} \)
\( 10^{-1} \)
\( 10^{0} \)
\( 10^{1} \)
\( 10^{2} \)

\( G (Pa) \)

\( 10^{-3} \)
\( 10^{-2} \)
\( 10^{-1} \)
\( 10^{0} \)

\( 10^{1} \)
\( 10^{2} \)
\( 10^{3} \)
\( 10^{4} \)

\( c (mg/mL) \)

These equations represent relationships between concentration and various properties:

\( \sigma_c \sim c^{0.68 \pm 0.22} \)
\( \sigma_c \sim c^{0.26 \pm 0.20} \)

Figure 4.7. The scaling of a) the plateau storage modulus, \( G_0 \) and b) the stress at onset of stiffening \( \sigma_c \) of agarose hydrogels as a function of concentration. Filled dots and the solid line represent the pre-strain protocol at 20 °C, open dots and the dashed line represent the amplitude sweep at 20 °C and crosses and the dotted line represent the amplitude sweep at 37 °C. The power laws against the concentration are presented with their 95 % confidence interval.

\( \frac{K}{K_{LVR}} \)

\( \gamma \)

\( \gamma_c \)

\( \gamma_c \sim c^{0.58 \pm 0.22} \)
\( \gamma_c \sim c^{0.26 \pm 0.20} \)

Figure 4.8. Normalized differential modulus, \( K \), as a function of strain, \( \gamma \). Inset shows the scaling of strain at the onset of stiffening, \( \gamma_c \), as a function of agarose concentration. a) in PBS at 20 °C, and at 37 °C adapted with permission from Publication IV. Copyright (2019) American Chemical Society. b) in H2O at 20 °C adapted with permission from Publication V. Copyright (2020) American Chemical Society.

arise from the enthalpic or entropic contribution [54, 81, 58, 24]. Therefore, the normal stresses of agarose hydrogels were studied in the publication V. Here, the negative normal stress is actually the negative apparent normal stress difference, \( N \), which was introduced in chapter 3.

All the agarose hydrogels showed negative normal force and stress when sheared (Figure 4.9) corresponding to slight contraction of the specimen when sheared. The rheometer simultaneously maintains a fixed gap size and the effect is seen as negative normal force and negative \( N \). The gels show negative normal
force already during gelation. Gelation has been shown to result in negative normal stress also in hyaluronic acid [19] and agar [75, 74]. The formation of networks and crosslinks creates internal stresses, which are seen as negative normal stresses. Gels at higher concentrations show higher absolute values of normal force and stress, as more fibrils are formed and more fibrils are involved in contracting the gel.

\[
F_z (N) = -0.7 \quad -0.6 \quad -0.5 \quad -0.4 \quad -0.3 \quad -0.2 \quad -0.1 \quad 0.05 \quad 0.1 \quad 0.15 \quad 0.2 \quad 0.25
\]

\[
2.5 \text{ mg/mL} \quad 5.1 \text{ mg/mL} \quad 7.5 \text{ mg/mL} \quad 10.1 \text{ mg/mL}
\]

\[
\frac{|N|}{\sigma} = 0 \quad 0.05 \quad 0.1 \quad 0.15 \quad 0.2 \quad 0.25
\]

\[
2.5 \text{ mg/mL} \quad 5.1 \text{ mg/mL} \quad 7.5 \text{ mg/mL} \quad 10.1 \text{ mg/mL}
\]

\[
\gamma
\]

**Figure 4.9.** a) Normal force as a function of shear strain, $\gamma$, in agarose hydrogels. b) The ratio between the absolute value of the apparent normal stress difference, $N$, and shear stress, $\sigma$, as a function of $\gamma$. Adapted with permission from Publication V. Copyright (2020) American Chemical Society.

The ratio between the absolute values of $N$, and $\sigma$ can be used to classify the thermal and athermal contributions [58, 24]. The magnitude of the normal stress cannot exceed the shear stress in a purely thermal model [54]. In Figure 4.9 b, the $N > \sigma$ resulting in a strong overshoot of $|N/\sigma|$ in low concentration agarose gels. Both of these findings support athermal network behavior [58, 24].

A negative $N$ is seen if there is a force that tries to pull the rheometer plates closer to each other when sheared. Even the surface tension of water resulting from the underfilling of the gap is observed as a negative normal force. Similarly, the overfilling of the gap causes a positive normal force. Therefore, great care was taken during the filling of the gap. Typically, slight overfill was aimed for, due to specimen contraction during the gelling.

Slight contraction of the agarose hydrogel is possible during shear if the water is not totally viscously coupled with the network and can move with respect to network and balance the pressure difference caused by the hoop stress. To study whether the water diffuses relatively fast we followed the method introduced by de Cagny et al. [29] (Figure 4.10). When oscillations take place both in the positive and negative shear direction, the intrinsically nonlinear property of the normal stress is easily seen. At low frequencies, the minimum of $N$ (the most negative value) is achieved at the peaks of shear strain or stress resulting in downward opening parabola in Figure 4.10 a) and b). When shear strain is zero, $N$ reaches its highest values.

At 0.1 rad/s the shear strain and stress are in the same phase. However, when
the frequency is increased to 10 rad/s a clear phase-shift is seen. There is also a much smaller phase shift between strain and $N$. The phase-shift between shear stress and $N$ results in the butterfly shape seen in Figure 4.10 c. At a higher frequency there is not enough time for the water to diffuse through and the gel can no longer contract similarly. If the frequency is increased, an upward opening parabola should appear. However, the data became too noisy above 10 rad/s and the upward opened parabola was not achieved.

4.2.4 Agarose Hydrogels as a Luminal Identity Preserving Scaffold, Publication VI

Cells in tissues are continuously exposed to different physical forces, including tension and compression forces, shear stresses, and hydrostatic pressures. The cells sense and adapt to physical forces by remodeling the microenvironment in response to the physical cues. Nowadays, it is widely recognized that mechanical cues together with biochemical signals regulate cell and tissue behavior. [20] During the past few decades the stiffness and viscoelastic nature of the scaffolding matrix has been shown to regulate the tissue phenotype [91, 70, 22, 135, 64, 31, 28].

Breast cancer is the number one cancer and cause of death of women in Finland [90]. Over 70% of breast cancers are luminal cell type expressing the estrogen receptor (ERα) [95]. Pre-clinical studies usually use cell lines for screenings to
see whether the cells will respond positively to the new treatment. The major problem is that cell lines do not represent well authentic patient cells and miss tissue heterogeneity. [27, 99] Similar problems lie in animal studies, as there are significant differences in the metabolism and immunology of different species [96]. Thus, many drug discoveries fail at the clinical trials stage [48]. Therefore, there is huge demand for reliable pre-clinical models. Figure 4.11 presents our pre-clinical model. It is based on explants from patients cultured in 3D hydrogel scaffolds. The patient-derived tumor explant culture (PDEC) systems are beneficial as they offer tumor-specific genetic and phenotypic heterogeneity, which have authentic tumor microenvironmental components. [97] PDECs are hence a step towards personalized medicine.

The luminal cells are located inside the mammary glands while basal cells form the outer layer (Figure 4.11). Paradoxically, in ex vivo cell culturing systems the luminal phenotype and (ERα) are both lost [42]. In publication VI, we show that the most scaffolding gels promote basal differentiation, while agarose is one of the hydrogels in which the luminal identity is preserved both in normal mouse and non cancerous patient tissues (Figure 4.12). Generally, basal cells are fast growing and typically dominate the cultures [42]. Here, we connect the change from luminal to basal cell identity to the presence of growth factors or cell adhesion sites which seem to promote basal differentiation.

The current models and protocols do not promote (ERα) expression and hormone receptor expression is lost already in short-term ex vivo cultures. [45, 121, 42] Publication VI demonstrates that the stiffness of the surrounding matrices plays a central role in preserving the (ERα)+. With agarose hydrogels it was possible to create bioinert stiff gels, which resulted in preserving the luminal identity as well as (ERα)+ in mouse tissues (Figure 4.13). Agarose hydrogels should have a storage modulus of 10 kPa (or an effective shear modulus of 30 kPa), which means agarose concentrations above 20 mg/mL.
**Results and Discussion**

**Figure 4.12.** Scaffolding matrix impacts on epithelial cell identity. The studied hydrogels were divided into basal promoting matrices, BMx, and luminal preserving matrices, LMx, based on the results. Immunofluorescence samples of normal mouse mammary tissue, MMEC, and normal human breast tissue, PDEC-N, were stained for CK8 (red) and CK14 (green) after 7 days culture in the matrices. The frequency sweeps obtained by oscillatory rheology show that the matrices varied from viscous fluid and soft gels all the way to stiff gels. Elastic modulus \( E \) is estimated from the absolute value of the complex modulus \( E = 2(1 + \nu)\left| G^\ast \right| \), where Poisson’s ratio \( \nu \) is 0.44. Adapted from Publication VI.

**Figure 4.13.** a) ER\( \alpha \) is preserved in agarose gels with concentrations above 20 mg/mL. The rheological frequency sweeps show the storage, \( G' \), and loss, \( G'' \), moduli of the studied gels. Adapted from Publication VI.

Mammary tissue in mice is mainly fat-containing and is biologically softer than the microenvironment for human breast stroma [33]. Human mammary epithelium is expected to need a higher stiffness to express \( \text{ER} \alpha+ \). We produced higher effective stiffness levels mechanically by pressing the culture with help of permanent magnets (Figure 4.14). The compression stress was calculated to be 37 kPa, which was estimated to cause an effective shear modulus \( |G^\ast| = 129kPa \). In compressed cultures, both the \( \text{ER} \alpha \) and p38 stress pathways are activated.
The current study suggests that the an effective shear modulus of the matrix of at least 10 kPa is required for activation of the stress pathway which leads to the expression of (ERα)+ in mouse tissues. Human tissues require almost 12-fold higher effective shear modulus to have a similar effect. Although strain-stiffening has been noted to be important in cell differentiation and proliferation [118, 28, 136], we could not conclude that strain-stiffening has an effect on the luminal (ERα)+ cell type.
This thesis presented two mechanically very different biomimetic materials: clay/polymer layered nanocomposite films and agarose hydrogels. Both materials consist of nanoscale components that self-assemble into macroscopic materials. In addition, two mechanical phenomena, i.e., toughness and strain-stiffening, were introduced. In nature, the both are connected to preserving the integrity of biological materials and preventing mechanical failures. The main focus, throughout the thesis, was on those mechanisms and structures that control the emergent mechanical properties.

The nacre-mimetic clay/polymer nanocomposite films prepared in publications I-III have a tensile strength and strain matching those of natural nacre. However, they have only half of the stiffness, since there is a larger fraction of reinforcing platelets in natural nacre. With current starting materials the amount of reinforcing clay cannot be easily increased, without compromising the properties. However, simply by using thicker reinforcing sheets (e.g. stacks of nonswellable clays) or clay platelets with a larger aspect ratio the fraction of reinforcement can be raised.

The functionalization of PDAMDAC-MTM nanocomposites with monophosphates in publications I and II led to synergistic improvements in strength, stiffness, strain, and work-to-failure in humid conditions. This suggests that the additional hydrogen bonding motifs can act as sacrificial bonds and allow dissipative deformation if enough plasticizing molecules, like water, are present. Yet, stiffness and strength were compromised in high humidity conditions.

In publication III, the effect of humidity was further studied with PVA-MTM nanocomposite films with intrinsic hydrogen bonding properties. Tensile testing and DMA analysis at different humidities revealed the connection of the glass transition temperature of the nanocomposite to the work-to-failure representing the toughness. Stable crack propagation and stress whitening were observed, which are indications of a macroscopic process zone and of dissipative sliding of the MTM platelets.

The observations made on the effects of humidity on the clay/polymer nanocomposites were valuable lessons for the design of future bioinspired materials. Some steps have already made towards developing more hydrophobic polymers.
Conclusions and Future Perspectives

and more effective architectures in functional nanocomposites [80]. However, tremendous efforts are still needed to design better polymers, including the control of $T_g$ and the implementation of energy dissipation mechanisms, which are activated only after a certain stress or strain level. Agarose hydrogels have been studied and used for over century. However, we have shown in publications IV-VI that there are still properties to be studied. The strain-stiffening, negative apparent normal stress upon shearing and helical twisting of the agarose fibrils were demonstrated for the first time. Our main findings support the fact that agarose hydrogels behave like subisostatic athermal networks. Firstly, simple size comparison of the persistence length and mesh size hinted that enthalpic stretching is mainly responsible for their mechanical behavior. Secondly, the differential modulus scales by 1 as a function of stress, thereby deviating from the universal $3/2$ scaling of thermal networks. Thirdly, the connectivity seems to dictate the strain onset of stiffening. Lastly, the apparent normal stress clearly exceeds the magnitude of shear stress when sheared.

Finally, publication VI presented an application of the agarose hydrogels as a scaffold for healthy mouse mammary tissue, as well as healthy and cancerous patient-derived breast explants. The agarose hydrogel is desirable scaffolding material for patient-derived luminal ERα+ breast cancer explants and those together offer a reliable pre-clinical model for drug development or personalized medicine. In addition, the publication showed the importance of the mechanical properties of the surrounding environment. The patent application considering the model was filed in April 2020. Overall, the findings on agarose hydrogels suggest to update the rheological studies of other polysaccharide-based gels consisting of semiflexible fibrils.

Biomimetic material design is an approach that can provide alternative ways of achieving lightweight structural materials. Understanding the mechanisms that prevent mechanical failure in biological materials is essential if more sophisticated properties like toughness, strain-stiffening or high fatigue resistance are desired. In addition, biomimetic processing methods based on self-assembly in mild conditions are more sustainable compared with traditional fabrication methods. Although some of the concepts and results presented here are based on previous research, in some cases dating back more than century, the biomimetic research field is rather young and active, and there are plenty of future challenges with regard to transferring ideas into practical usage. Biomimetics is, for example, moving towards solutions for the full-life-cycle -degradation and reuse of the materials [76], which presents major challenges for the starting materials.
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The field of biomimetics aims to borrow nature’s well-adapted design strategies, structures and functions to solve material engineering problems. This thesis focuses on two nature-inspired systems: (i) tough and strong nacre-mimetic nanocomposites, and (ii) strain-stiffening biopolymer hydrogels and their application for cell culturing. Both systems consist of nanoscale components. Their mechanical properties and connection to structure are studied. Based on a biomimetic materials design approach, the first part of this thesis illustrates a simple method to control the mechanical properties of layered clay-polymer nanocomposites. The second part presents insights into the fibril network mechanics of agarose hydrogels. Finally, the last publication introduces a reliable agarose-based preclinical model, which can be used as a platform for breast cancer drug development and personalized cancer therapy.

Biomimetic materials design towards tough nanocomposites and strain-stiffening hydrogels

Lahja Martikainen

Aalto University
School of Science
The Department of Applied Physics