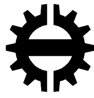

TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

CHRISTINE GERING

FUNCTIONALIZATION OF THE POLYSACCHARIDE HYDROGEL GELLAN GUM FOR TISSUE ENGINEERING APPLICATIONS

Master of Science thesis

Examiner: Prof. Minna Kellomäki and
Janne Koivisto
Examiner and topic approved by the
Faculty Council of the Faculty of
Engineering Sciences
on 9th December 2015

ABSTRACT

Christine Gering: Functionalization of the polysaccharide hydrogel gellan gum for tissue engineering applications
Tampere University of Technology
Master of Science Thesis, 50 pages, 8 Appendix pages
May 2016
Master's Degree Program in Materials Science
Major: Polymeric Materials
Examiner: Professor Minna Kellomäki and MSc(Tech) Janne Koivisto

Keywords: hydrogel, gellan gum, functionalization, tissue engineering, avidin-biotin binding, mechanical testing, carbodiimide coupling

Hydrogels have for long been a promising class of materials for tissue engineering applications. Essentially, hydrogels form a scaffold-like support structure for cells and provide an aqueous environment. This artificial environment mimics natural tissues and allows for the research on cells and the effect of factors onto cells. Furthermore, hydrogels can be used in regenerative medicine for cell delivery to damaged tissue. The herein studied hydrogel material is the polysaccharide gellan gum, which has been developed as a food additive, but has recently been proposed as a suitable tissue engineering material.

Although most hydrogel materials are biocompatible and do not negatively affect cell growth, they are also biologically relatively inert. To combat this situation, various approaches have been described in the literature to functionalize hydrogels with an abundance of different bioactive molecules through the means of various chemical strategies. Likewise, gellan gum hydrogels have been used successfully in cell culture, but satisfying cell adhesion and response have not been achieved.

This thesis work describes the chemical functionalization of gellan gum and the covalent binding of the protein avidin to the gellan gum. Avidin is a tetrameric protein which binds biotin with high specificity and affinity. This allows for the convenient and flexible modification of the gellan gum network with biotin-labelled compounds, notably biotinylated ligands for cell attachment and signaling. Therefore, sodium purified gellan gum was successfully functionalized with avidin over carbodiimide coupling. Self-supporting gel samples could be created from the functionalized gellan gum. Commercial gellan gum was purified with an established method and its elemental composition was analyzed with atomic absorption spectroscopy. The covalent coupling of avidin was verified with gel electrophoresis, while its functionality was determined with fluorescence spectroscopy. Hydrogel samples were formed with calcium and bioamines and the mechanical properties of the gels were examined with compression testing.

The results verify that the presented approach offers a mild functionalization that does not disturb hydrogel gelation or the avidin-biotin binding. Further work is required to improve the cross-linking and gel sample production, in order to achieve consistent results of parallel samples with good gel structure and desired suitable mechanical behavior. The next steps will be to discern a suitable biotinylated bioactive cue, such as biotinylated RGD, and test the ability of the functionalized gellan gum to serve as a cell culture matrix.

PREFACE

This thesis was written at the Tampere University of Technology in the department of Electronics and Communication Engineering. The research for this thesis project was conducted in the Biomaterials group directed by Professor Minna Kellomäki.

I wish to thank my supervisors Janne Koivisto and Minna Kellomäki for providing such an interesting and challenging thesis project to me and overseeing the progress of my work. I want to also acknowledge Jennika Karvinen and Jenny Parraga from the hydrogel group for their practical support and valuable comments. I also wish to thank Jonathan Massera for assisting with the atomic absorption spectroscopy (AAS).

Further I want to express my very special thanks to Jenni Leppiniemi and Vesa Hytönen at the Protein Dynamics research group of BioMediTech, UTA. Not only for providing resources and the facilities at FinnMedi, but also for taking the time to carry out the gel electrophoresis with me and actively supporting my work.

Tampere, May 05, 2016

Christine Gering

CONTENTS

1. INTRODUCTION 1
2. THEORETICAL BACKGROUND 3
  2.1 Gellan gum 3
    2.1.1 Gelation of gellan gum 4
    2.1.2 Properties of gellan gum 7
  2.2 Biomaterials as cell environment 8
    2.2.1 Biochemical environment 9
    2.2.2 Mechanical and physical cues 12
  2.3 Functionalization strategies 13
    2.3.1 Physical and covalent coupling strategies 13
    2.3.2 Functionalization of the polymer or final hydrogel 16
    2.3.3 Characterization of functionalized hydrogels 17
  2.4 Gellan gum functionalized with avidin 21
    2.4.1 Avidin and the avidin-biotin binding 22
3. EXPERIMENTAL PART 25
  3.1 Sample preparation 25
    3.1.1 Purification of gellan gum 25
    3.1.2 Functionalization of sodium purified gellan gum 25
    3.1.3 Gel sample preparation 26
  3.2 Characterization methods 26
    3.2.1 Atomic absorption spectroscopy 27
    3.2.2 Fluorescence spectroscopy 27
    3.2.3 Gel electrophoresis 28
    3.2.4 Compression testing 28
4. RESULTS AND DISCUSSION 29
  4.1 Initial considerations 29
  4.2 Success of functionalization 31
  4.3 Gelation properties and compression testing 35
5. CONCLUSIONS 41
REFERENCES 43

Appendix A: Table of functionalized hydrogels in the literature 1
Appendix B: Table of used materials and chemicals 3
Appendix C: Effects of solvents on Gellan gum and avidin 5
Appendix D: Calculation of carboxyl groups in gellan gum 6
Appendix E: Protocol for Purification of gellan gum 7
Appendix F: Protocol for Functionalization of sodium-purified gellan gum 8

LIST OF FIGURES

Figure 1. *Chemical structure of gellan gum (α-L-Rha, β-D-Glc, β-D-GlcA, β-D-Glc) with highlighted carboxyl group [7]. 3*
Figure 2. *Gelation mechanism of gellan gum (based on [7]). 6*
Figure 3. *Chemical structures of the bioamines spermine (SPM) and spermidine (SPD) used for the gelation of GG. 7*
Figure 4. *Categories of functionalization strategies. 13*
Figure 5. *Carbodiimide coupling strategy with EDC and NHS. 14*
Figure 6. *Schematic representation of gellan gum network with covalently bound avidin. The exemplary biotinylated compound "C" is coupled to the network over the avidin-biotin binding. 21*
Figure 7. *Avidin structure as ribbon diagram [66](A) and the chemical structure of biotin (B). 23*
Figure 8. *Schematic of avidin-biotin binding with a single type (a) and a mixture (b) of biotinylated species. 23*
Figure 9. *Schematic representation of avidin tetramer separation into monomers [67], while one of the monomer blocks is coupled to the gellan gum chain (blue line). 24*
Figure 10. *Measuring set-up for compression testing with the Bose instrument. 28*
Figure 11. *Chemical mechanism of the activation of GG with EDC and NHS and subsequent functionalization with avidin (primary amine). 30*
Figure 12. *Fluorescence spectrum of NaGG-avidin. Dotted line: pure NaGG-avidin; Solid line: NaGG-avd and B5f, quenched fluorescence; Dashed line: NaGG-avd with added biotin, before the B5f is added. 32*
Figure 13. *Fluorescence spectrum of different substances with identical amount of B5f. Dotted line: HEPES (solvent of the other gellan gum samples); Solid line: NaGG-avidin (0.1 wt%, same as shown in Fig. 12); Dashed line: NaGG +avidin (0.1 wt%). 33*
Figure 14. *Image of the SDS-PAGE gel, oriole stained, 200 V for 30 min. 34*
Figure 15. *Gel samples from NaGG-avidin in HEPES (50 mM, pH 6.5) with sucrose (10%). 36*
Figure 16. *Photograph illustrating the transparency of NaGG-avidin gel samples in HEPES/sucrose pH 6.5 with different gelation agents. 36*
Figure 17. *Single result stress-strain curves of NaGG gel samples with SPM, SPD and calcium. 37*
Figure 18. *Stress-strain curves of the following gel sample: GG (SPM 0.35 mg/mL) 18.1 kPa, GG (SPD 1.0 mg/mL) 30.2 kPa, NaGG (SPM 0.35 mg/mL) 20.2 kPa, NaGG-avidin (SPD 1.0 mg/mL) 52.0 kPa. 38*
Figure 19. *NaGG-avd samples cross-linked with $CaCl_2$ (0.35 mg/mL, 116.4 kPa), SPM (0.75 mg/mL in HEPES pH 5.5, 21.3 kPa) and SPD (1.0 mg/mL in HEPES pH 5.5, 7.1 kPa). 39*

LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
A	Area
AAS	Atomic absorption spectroscopy
B5f	Biotin-5-fluorescein (biotinylated fluorescence dye)
BMCS	Bone marrow stromal cells
CNCA	Charge neutralized chimeric avidin, also abbreviated as nChiAvd
Da (kDa)	Dalton (kilo Dalton)
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
F	Force
FRAP	Fluorescence recovery after photobleaching
G'	Storage modulus
G''	Loss modulus
GG	Gellan gum
GI	Gastrointestinal
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
l, l_0	Height, initial height
MA	Methylacrylate
NaGG	Sodium purified gellan gum
NaGG-avidin	Avidin functionalized sodium purified gellan gum
nChiAvd	Charge neutralized chimeric avidin
NHS	N-Hydroxysuccinimide
pI	Isoelectric point
RGD	Arginylglycylaspartic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate - poly(acrylamide) gel electrophoresis
SI system	Système international d'unités, International System of Units
SPD	Spermidine (1,8-Diamino-4-azaoctane, N-(3-Aminopropyl)-1,4-diaminobutane)
SPM	Spermine (N,N′-Bis(3-aminopropyl)-1,4-diaminobutane)
tan δ	Viscoelastic loss factor
TUT	Tampere University of Technology
UTA	University of Tampere
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XPS	X-ray photoelectron spectroscopy
ε	Strain
σ	Stress

1. INTRODUCTION

The field of tissue engineering is an ambitious discipline to study and create artificial, functional tissues comprised of a scaffold, cells and bioactive compounds. The scaffold is made from biomaterial and is one of the most crucial factors, as it serves as an artificial support matrix for the cells and forms the basis of the biological tissue analogue [1]. These artificial matrices need to mimic the natural cell environment in their mechanical properties and provide biological cues to allow for cell-matrix-adhesion and signaling [2] [3] [4]. A positive cell response is required for the proliferation and behavior of cells equal to their development in natural tissue. While some of the required cues are soluble and not bound into the matrix, other cues such as adhesion ligands must be firmly attached to the scaffold in order to provoke ligand-receptor signaling [2] [4].

The application area of engineered artificial tissues and cell cultures includes, on one hand, the study of cells and tissues to mimic biological tissue, as well as studying the effect of external factors, for example preclinical screening of drug candidates [5]. On the other hand, these cell-matrix systems can be employed in regenerative medicine as support structures for cell delivery to damaged tissues, in order to improve cell survival rate upon implantation. The material requirements for a successful cell support include bio- and cytocompatibility, a suitable biochemical environment where soluble cues like growth factors and adhesion factors are present, as well as appropriate mechanical properties [2].

Hydrogels are hydrophilic polymer scaffold that significantly swell in water, and the water content of a hydrogel can be well above 90%. They have for long time been considered outstanding material for tissue engineering [6]. Due to their hydrophilic nature they can provide a suitable aqueous environment for biological molecules. Gellan gum is a polysaccharide capable of forming hydrogels and it provides several advantages over other materials used for tissue engineering applications. Gellan gum is produced by bacteria, and thus can be classified as natural polymer. As opposed to biomaterials from mammalian sources, like collagen, the bacterial source avoids issues of disease transmission [1]. Finally, gellan gum can form transparent, self-supporting gels in the presence of mono- or divalent cations [7].

Although polysaccharides, like many other hydrogel materials, show good biocompatibility and are not cytotoxic, most types of polysaccharides are not very bioactive and do not provide the necessary cues for cell differentiation, proliferation and attachment [8]. It may also be of interest to stimulate other specific cell functions, such

migration and morphology, or beating of cardiomyocytes, neurite outgrowth of neural cells [3] [4]. Therefore, the modification and functionalization of hydrogels is required.

For tissue engineering purposes, biomaterials need to be designed not only in their biochemical properties, but also in their physical and mechanical material properties. The modification and functionalization of hydrogels has been widely discussed throughout the literature and it has been argued that a rational design of hydrogels for tissue engineering applications needs to be pursued [2]. The mechanical and physical properties of hydrogels are commonly modified by altering the monomer composition, polymer concentration or changing the gelation agent [7] [9]. To enhance the biochemical makeup experienced by cells, hydrogels can be equipped with bioactive compounds via covalent coupling or physical immobilization [all in Appendix A]. However, it is required that the functionalization does not alter biocompatibility of the material or induce cytotoxicity, and neither significantly deteriorate mechanical stability of the hydrogel.

This thesis project is part of the Human Spare Parts project by the BioMediTech Institute in Tampere. Due to collaboration with two different stem cell research groups of the University of Tampere ("Heart Group" and "Neuro Group"), the focus application for modified gellan gum hydrogels are for neural cell types and cardiomyocytes. Although the discussion will be kept mostly general, in some cases it will be referred to specifically these cell types.

Within this thesis the functionalization of gellan gum and hydrogel materials in general will be explored. The protein avidin is coupled to the gellan gum and the gel forming ability and mechanical properties of the resulting hydrogel are investigated. The theoretical part of this thesis will give an introduction to gellan gum, by describing its properties and gelation mechanism. In conjunction the approach to characterize hydrogels for medical applications will be described briefly. Secondly an overview of the requirements for cell environments are given, including the biochemical and mechanical properties, as well as a discussion about three-dimensional (3D) cell culture. Thirdly, the theoretical background will be considerations about different functionalization strategies for hydrogels. Finally, the approach of creating a functionalized and cell-responsive gellan gum hydrogel will be detailed. The research part will describe the purification of gellan gum, the functionalization of gellan gum with avidin and also give a description of the employed characterization methods. As for results of the project, the initial conditions for the gellan gum functionalization will be discussed, followed by the results and argument over the success of the functionalization. Furthermore, the mechanical properties of the resulting hydrogel are examined. Finally, conclusions about the project and applicability of the created gellan gum-avidin system will be drawn.

2. THEORETICAL BACKGROUND

2.1 Gellan gum

The polymer investigated in this thesis is gellan gum (GG), which is distributed under the tradename “Gelzan” by CP Kelco U.S., Inc. GG is an anionic exopolysaccharide, where one repeating unit is composed of the four saccharides L-rhamnose, D-glucopyranose, D-glucuronic acid and D-glucopyranose. The structural formula of one such repeat unit is shown in Fig. 1 [7]. The carboxyl group in the glucuronic acid is emphasized with color, because it provides a convenient opportunity for the chemical modification of GG [10].

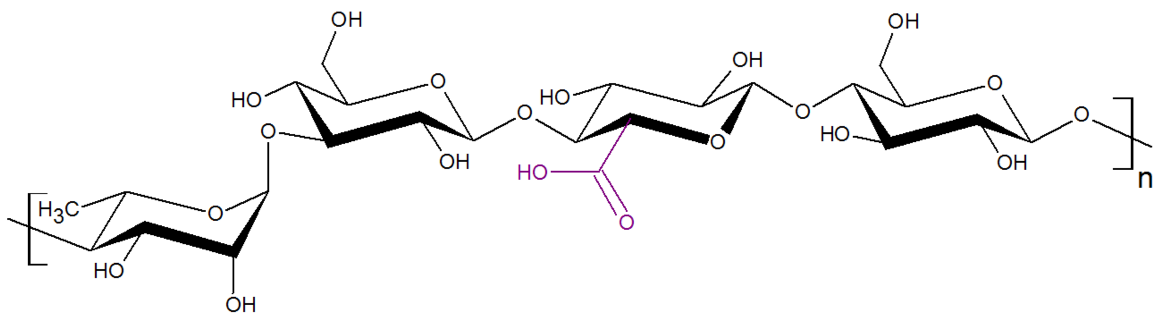

Figure 1. *Chemical structure of gellan gum (α-L-Rha, β-D-Glc, β-D-GlcA, β-D-Glc) with highlighted carboxyl group [7].*

The polymer is produced by the bacteria *Sphingomonas elodea* (ATCC31461) in an aerobic process with relatively high yield. Polymers produced by bacteria offer the advantage of high tissue compatibility common to naturally derived products [11]. In contrast to animal-derived biomaterials, for example collagen or the polysaccharides hyaluronan and glycogen, gellan gum can be purified and is available commercially as a product free of endotoxins [12]. Collagen is extracted from connective tissue and needs to be sterilized for further biomedical application, but due to its intricate structure can still evoke an acute immune response [1].

After fermentation, the substance is treated in a hot alkaline bath, which removes the naturally occurring acetyl groups from the glucose monomer, to yield the de-acetylated form, or “low-acyl” form, of GG [7]. Within this thesis GG refers to the low-acyl form of gellan gum. The average molecular mass of GG is 500 kDa, as established through static light scattering method [11], from which an estimated amount of 700 repeat units in one GG chain can be derived (calculation in appendix D) [7].

Initially GG was developed for the food industry and intended to be used as stabilizer and thickening agent [12], much like alginates and gelatins. It was not discovered as though by chance, but identified through a targeted screening effort by the company Kelco, looking specifically for polymers produced by soil and water bacteria [11]. GG was one

of the few materials discovered through this program which were then found to have commercial potential. Other applications include GG as additive for cosmetics, lotions and toothpaste. The advantageous properties of GG as thickening agent in these applications include an increased flavor release and good gel stability over a wide temperature range [13].

Next to food applications, GG has been extensively investigated for medical applications [14]. It has been used in various drug formulations as a release matrix, or as a component of the release matrix [15], [16], [17]. GG offers a range of favorable properties that can aid the controlled and prolonged release of the active pharmaceutical ingredient. The release from the matrix depends on a complex mechanism mediated by swelling, diffusion and erosion of the system. Other advantageous properties of GG include its ability of *in situ* gelation when in contact with low pH or cations, as well as its potential to adhere to mucosa and other biological surfaces [16]. Drug encapsulation systems for oral delivery can be designed to either be administered in solid form and slowly dissolve in the gastrointestinal (GI) tract or be taken up in non-gelated form and create gels *in situ* when in contact with the acidic environment of the GI tract. Formulations for nasal delivery have the potential to evoke a systemic action of the drug, while avoiding the GI tract which can have negative effects on the drug itself. For nasal delivery the mucoadhesion of GG is crucial to obtain a sustained release. Ophthalmic delivery systems, i.e. delivery through the cornea of the eye, exploit the gel formation of GG when in contact with tear fluid. The gel will adhere and thus enhance the bioavailability of the drug [16]. A well-established ophthalmic drug delivery system using GG is the Timoptic-XE®, which has been on the market since 1993. It was reported that the formulation increases the bioavailability of the drug timolol up to four times [18].

2.1.1 *Gelation of gellan gum*

The most crucial property of GG is, of course, its ability to form hydrogels with adequate mechanical properties and under adequate thermal conditions. Traditionally GG is cross-linked with divalent cations, typically calcium ions, in order to form physical hydrogels. The commercial formulation of Gelzan™ contains sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium ions (Ca^{2+}), thus an aqueous solution of GG can be gelated by heating and subsequent cooling [7]. Hydrogels can be created from as low as 0.1% (w/w) GG solutions [14], however those low concentrations form weak, non-self-supporting gels. The calcium ions present in the formulation serve as the primary means of gelation by complexing carboxylate groups of adjacent GG chains. Nevertheless GG can also be cross-linked with monovalent ions, such as Na^+, K^+ and also cationic compounds such as tetramethylammonium (Me_4N^+) [7] or cationic organic compounds such as spermine (SPM) and spermidine (SPD), as presented in this project.

In order to study the gelation of GG, the polymer can be purified to either the free acid form [19] or monovalent cation form, usually sodium-purified GG (NaGG) [20], [10].

Table 1 shows an elemental analysis of counter-ions for food grade and purified GG (from [10]).

Table 1. *Cation content of gellan gum in the literature [10].*

Element (wt%)	Na^+	K^+	Ca^{2+}	Mg^{2+}
food grade GG	0.6 ±0.1	4.5 ±0.2	1.2 ±0.1	0.11 ±0.01
Na-purified GG	2.5 ±0.1	1.0 ±0.1	<0.06	<0.03

Gelation can be achieved with any type of cationic species, however the concentration required to form true gels varies greatly with different cations. Divalent cations from group II (Ca^{2+}, Mg^{2+}) form the strongest gels at low concentrations, whereas monovalent cations from group I need much higher concentration to form similar gels. High concentrations of organic cations, such as Me_4N^+ studied by Morris et al., are able to create only weak gels [7].

In order to understand the gelation and network formation of GG, the process can be separated into different phases (refer to Fig. 2). At first, when GG is dissolved in an aqueous medium and warmed, the polysaccharide chains exist as disordered coils in solution (a). Upon cooling, GG adopts a double-helix structure (b) regardless of counter-ions present in the solution. This double helix has been described as a three-fold, left-handed and double-staggered helix, with a pitch of 5.64 nm [7]. Separate helices are connected through linear segments of the GG chain, which are approximately 150 nm long. Under non-gelling conditions, for example with Me_4N^+ as counter-ions or low concentrations of Na^+, double helices and linear segments form long filaments. Although these filaments are not aggregated or directly connected, weak gel properties may be observed, mostly due to branching of the filaments. Because GG is an anionic polysaccharide, with a number of carboxylate groups, the helices have a negative net charge and thus repulse each other. With the addition of cationic species to the solution, aggregation of the double helices occurs and a continuous network is formed (c) [7].

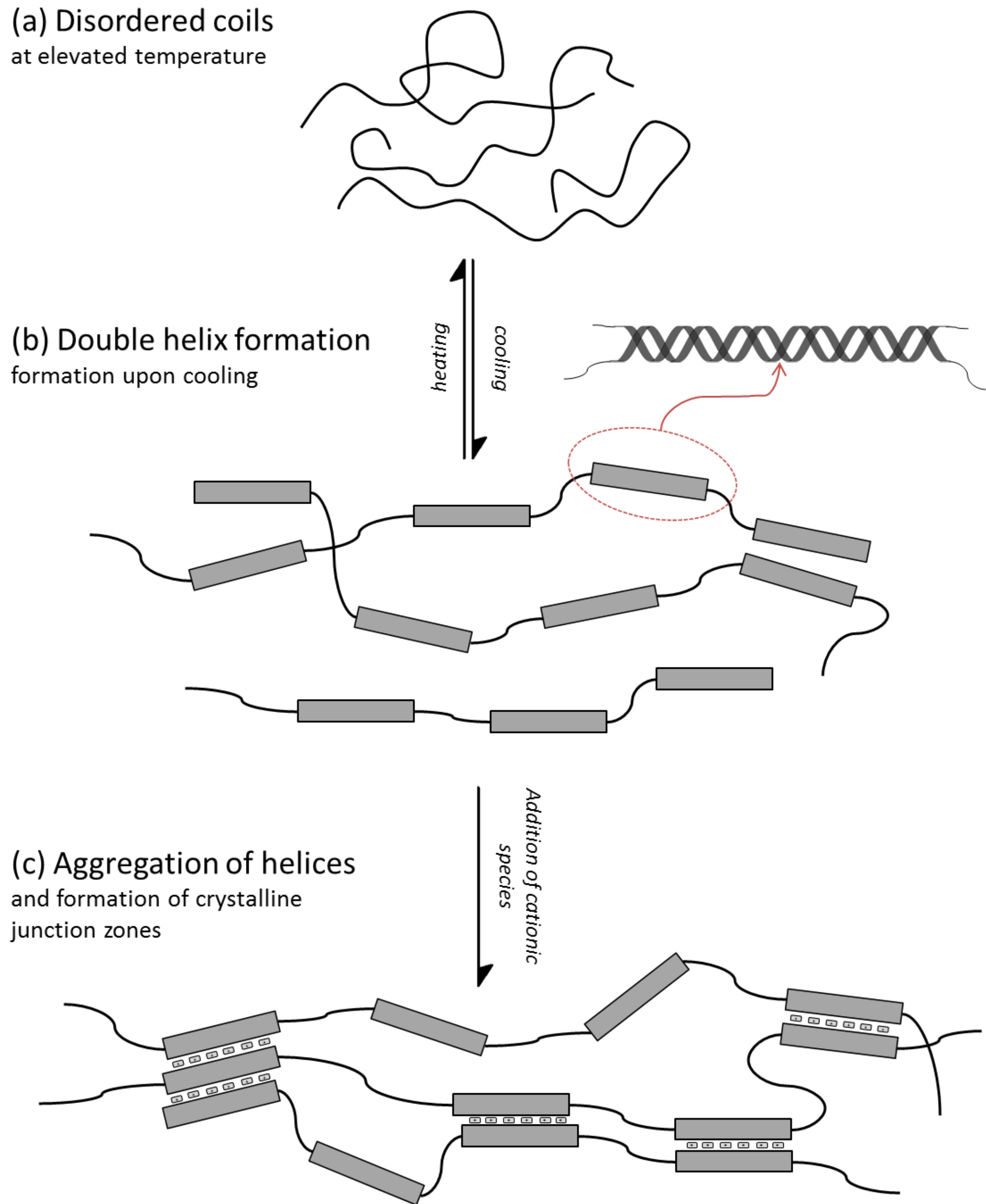

Figure 2. *Gelation mechanism of gellan gum (based on [7]).*

The mechanism of helix aggregation is, however, distinct for the different cationic species capable of gelating GG. Small, monovalent cations, such as Na^+ and K^+, reduce the helix repulsion by coordination with carboxylate groups on the helices. Similarly, other monovalent compounds like Me_4N^+ reduce the helix repulsion, but only via charge screening, because they are not able to form coordination complex with the carboxylate groups. This explains why higher concentration is needed in order to achieve aggregated clusters of helices. Finally the divalent cations of group II metals, like Ca^{2+} and Mg^{2+}, are capable forming GG gels by direct bridging between two carboxylate groups of neighboring helices [7]. The use of sucrose solution as solvent of GG promotes the conformational ordering into this helix structure and also facilitates gelation [7].

In this project the cross-linking is carried out with multivalent bioamines, namely spermine (SPM) and spermidine (SPD). These bioamines are multi-charged endogenous molecules; their chemical structure is shown in Fig. 3.

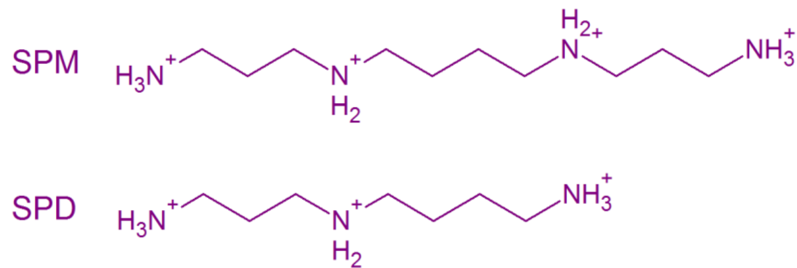

Figure 3. *Chemical structures of the bioamines spermine (SPM) and spermidine (SPD) used for the gelation of GG.*

At physiological pH they are fully protonated [21] and their gelation efficacy for GG has been proven in the literature [17]. The motive to use bioamines for the gelation of GG is to avoid an excessive amount of Ca^{2+} in the final gel, which is expected to negatively influence cell culture applications. The effect of Ca^{2+} concentration on cell culture has been studied an alginate hydrogels by Cao et al. [22]. Although there are several factors that affect the cell survival, it was found that an elevated Ca^{2+} content over a longer time period is detrimental for the cell culture [22]. Ultimately it would be beneficial to control the Ca^{2+} concentration rather through the applied culture medium, than it being determined by gelation requirements.

2.1.2 *Properties of gellan gum*

Gellan gum offers a range of properties which make it an excellent candidate as hydrogel for tissue engineering purposes. Next to its gelation characteristics and bacterial source, the mechanical, optical and mass transport properties should be considered [23].

GG is a viscoelastic material, with its mechanical and rheological properties strongly depending on the employed gelation agent and gelation circumstances, for example temperature. The mechanism of gelation and effect of different cationic species is described in chapter 2.1.2. Table 2 lists a range of examples of different solvent and gelation agents for GG in the literature. The reported modulus varies greatly, but roughly spans the values for soft tissues within the human body (further discussion about this in chapter 2.2.2). Aside from mere compression strength, GG shows a peculiar compression behavior depending on the speed the compressive force is applied. The gels will break under rapid compression, but retain their shape once the strain is released, whereas exudation of water and thinning can be observed under slow compression. Unless badly fractured, the gels will return to their original volume and height, when they are soaked in water over a period of time [7].

Table 2. *Moduli of different GG gels in the literature.*

Gelation agent	Concentration of gelation agent	GG concentration	Young's Modulus (E)	Reference
Ca^{2+}	9 mM	0.5 wt% H_2O	19.3 kPa	[24]
Na^{+}	280 mM	0.5 wt% H_2O	12.3 kPa	[24]
Na^{+}	100 mM	1.3 wt%	~110 kPa	[7]
Na^{+}	100 mM	0.5 wt%	~16 kPa	[7]
Spermine	0.24 mM	0.5 wt% sucrose	21.4 kPa	[25]
Spermidine	0.95 mM	0.5 wt% sucrose	21.9 kPa	[25]

A substantial advantage GG has over other hydrogel materials, such as nanocellulose, is its outstanding transparency. When dissolved in a suitable solvent, and even in gelated form, GG is colorless and very clear. Good optical properties like these are required when GG is used as cell matrix material in disease modelling or developmental biology applications, in order to study the cells with conventional microscopic methods [12]. In contrast to other polysaccharides, such as agarose, GG does not inhibit the enzymatic action of polymerase, which means that PCR can be carried out to identify markers of gene expression of the cell DNA [12].

Other properties of GG that are relevant for cell support include the diffusion within the hydrogel and the mobility of water. The majority of water in GG is free water and not bound to the polysaccharide backbone, thus it has the same mobility as free water in solution [12]. This is, of course, a crucial factor for the diffusion and transport of nutrients and waste products. Furthermore, GG is considered biocompatible and non-toxic [12].

2.2 Biomaterials as cell environment

The technique to grow cells in the laboratory in an artificial environment has been developed from the beginning of the 20th century. In order to reflect better the circumstances *in vivo*, many improvements of the original technique have been made since then [5]. These improvements include for example the refinement of the culture medium from blood plasma to synthetic plasma, as well as the development of the substrate material from glass dishes to polymers and coated surfaces. The significance to regulate the supply of required cues and factors for various cell functions through the culture medium and substrate has been acknowledged. Finally the aspects of 3D cell culture are being investigated in more detail in recent years [5]. Cells *in vivo* are evidently surrounded by tissue and extracellular matrix (ECM) in all spatial directions, whereas *in vitro* studies traditionally observe cells on 2D surface. This has led to conflicting results between *in vitro* and *in vivo* studies [5].

An artificial cell niche needs to be developed that allows for viability, attachment, proliferation, differentiation and migration of the considered cell type *in vitro*. Therefore, materials in cell culture need to be designed regarding the two interdependent factors of adhesiveness and substrate stiffness. The biochemical makeup of the material regulates how well a cell can adhere to the substrate. Effectively, the ECM can be mimicked by equipping the artificial growth matrix with macromolecules like proteins and proteoglycans, soluble cues such as growth factors and cytokines, and adhesion ligands, for example the peptide sequence RGD (refer to 2.2.1) [2]. Furthermore, and essential from the tissue engineering point of view, the cell environment must be mimicked also in the physical and mechanical properties, including rigidity, biocompatibility of the gelation mechanism, degradation behavior and mass transport (refer to 2.2.2) [2].

The goal of tissue engineering for cell culture is to guide cell fate in a reliable and controlled manner for *in vitro* modelling and *in vivo* medical applications. This means that tissues can be engineered for wide range of application ranging from basic cell research to treatment of injuries, which all rely on high similarity to the corresponding natural tissue. The similarity regards the biochemical and physical properties of the material on the one hand, and on the three-dimensionality of the system, fully surrounding the cells. Thus employing a so-called 3D cell culture allows for the study of cell-cell interactions, including attachment and signaling [26]. Furthermore, cell culture models which closely resemble the biological cell environment are beneficial for drug screening, in order to determine the clinical relevance of drugs. Reliable results from the *in vitro* phase of pharmaceutical research will decrease the cost for the *in vivo* phase and animal models [5]. The ultimate trial for three-dimensional (3D) engineered tissues is the implantation of growth matrices seeded with cells to heal tissue defects of living patients *in vivo*. The matrix has to take up the injured cavity to protect and support the cells in the early phase of wound healing [27].

Many authors stress the fact that a majority of the cell-matrix interactions and effect of other cues are still not fully understood. So far it has been pointed out by different groups that different biochemical and physical cues, as well as combinations thereof, have a different effect on different cell types. Furthermore the origin and shape of the cells as wells as the adhesion ligand density within the material varies [2] [4] [28] .

2.2.1 *Biochemical environment*

The principle mechanism of cell communication and adhesion to surfaces is the integrin receptor-ligand signaling pathway. Integrins are a class of transmembrane receptors with extracellular and intracellular domains, which recognize extracellular cues and communicate information into the cell, actively mediating and regulating cell processes [29]. These extracellular cues can be soluble, e.g. cations, or attached to the growth matrix, like extracellular matrix (ECM) ligands [4]. It is exactly these ligands, respective their nature and occurrence, which are of great interest when designing artificial cell

matrices. Table 3 summarizes different classes of bioactive compounds which have been studied for the guidance of cell behavior in artificial matrices. The list is certainly not exhaustive, but tries to give an overview and demonstrates that a majority of the bioactive compounds are derived from components of the ECM.

Table 3. *Bioactive compounds used for functionalization of biomaterials in the literature.*

Category	Compound	Derived from	Source
Peptide sequences	RGD (cyclic-RGD)	fibronectin, vitronectin, laminin and collagen type I	[4]
	REDV	fibronectin	[27]
	YIGSR, IKVAV, RYVVLPR and RNIAEIIKDI	laminin	[27] [30]
	QHREDGS	Angiopoietin Ang-1	[31]
Proteins	Collagen		[32]
	Fibronectin		[32]
	Nephronectin		[32]
	Laminin	glycoprotein of basal lamina	[32]
	Gelatin	collagen	[32]
Growth Factors	Vascular endothelial growth factor (VEGF)		[32]
	Granulocyte colony-stimulating factor (G-CSF)		[32], [33]
	Stromal-derived growth factor (SDF-1)		[32]
	Leukaemia inhibitory factor		[32]
	Insulin-like growth factor (IGF-1)		[32]
	Erythropoietin (EPO) (hormone)		[34]
(Synthetic) chemicals	Dimethyl sulfoxide (DMSO)		[32]
	All-trans retinoic acid (RA)	Vitamin A	[35]
	Dynorphin B	naturally occurring kappa-opioid	
	Ascorbic acid	Vitamin C	[36]
	5-aza-20-deoxycytidine (5-aza-dC)		[32]
	poly-L-lysine		[3] [37] [38]
Antibodies	Nogo receptor antibody (NgR-Ab)		[37]

Peptide sequences used to functionalize artificial cell matrices are the binding motifs of adhesion proteins, such as laminin and fibronectin [32]. The most abundant peptide sequence employed in the literature to functionalize substrates for cell culture is arginyl-

glycyl-aspartic acid (RGD). This short peptide consists of only three amino acids and has been identified to be key motif for integrin-ECM adhesion. Owing to its size and chemical structure, RGD is a good candidate for scaffold functionalization and indeed many studies claim a positive effect on cell adhesion and proliferation for a wide variety of matrix materials [8] [10] [13] [39] [40] [41] [42] (also refer to Appendix A). RGD-modified matrices appear to be feasible for a large range of cell types, however in some cases negative effects have been observed. For instance, Connelly et al. show that the chondrogenesis of bone marrow stromal cells (BMSC) is inhibited in RGD-modified alginate and agarose hydrogels. Nevertheless these RGD-modified hydrogels do provide adhesion and cell viability [28].

The incorporation of whole proteins is a logical step forward from using peptide fragments. A protein will be able to present the required binding motif in the correct conformation and spatial arrangement, but can potentially interact also with other cell receptors and promote additional cell responses. However random protein folding may be a problem, which can lead to the blocking of receptor binding sites. The proteins investigated for matrix functionalization in the literature are ECM components, such as collagen, laminin, fibronectin and nephronectin. With very large proteins, or a similar ratio of protein to polymer, the matrix systems can also be considered composite materials of polymer and protein [32].

Growth factors are small polypeptides which actively guide cell development and are able to induce angiogenesis or delay apoptosis. In the ECM they are present as soluble, slowly diffusing cues with a relatively short lifespan. Thus it is of great advantage if they are covalently attached to the artificial scaffold, which protects from fast inactivation or overcomes limitations of slow diffusion [32].

Some other chemicals, that do not belong into any of the other categories and are not necessarily of natural origin, have also shown a positive effect for cell development. Synthetic chemicals have the advantage of a defined chemical structure and purity, as well as greater stability and longer shelf life, however it must be considered that they may impair the biocompatibility for *in vivo* applications. Compounds such as ascorbic acid, retinoic acid, dimethyl sulfoxide (DMSO), and dynorphin B have been reported to be capable of inducing cardiomyogenic differentiation of embryonic stem cells [32] [35]. Poly-L-lysine is routinely used in 2D cell culture as surface coating in order to support the attachment of neural cells [38]. It creates a positive charge on the surface and thus mediates cell adhesion by regulating the charge. Pan et al. have successfully functionalized porous hyaluronic acid hydrogels with poly-L-lysine and demonstrated the adhesion and proliferation of neural progenitor cells (NPC) [37]. In the same article, the authors show the usefulness of incorporating the Nogo receptor antibody (NgR-Ab) into the hydrogel. In contrast to the plain hyaluronic acid hydrogel, as well as the poly-L-lysine modified hydrogel, the antibody-modified hydrogel supported the differentiation of NPCs into neurons and astrocytes [37].

2.2.2 *Mechanical and physical cues*

As briefly mentioned earlier, cell adhesion and proliferation are inevitably coupled to the substrate mechanical properties, most notably the stiffness of the matrix material. Mechanotransduction is the phenomenon that describes how the mechanical properties of the substrate affect the structural integrity of the cytoskeleton through the application of tension. Integrins will attach to the provided ligands of the matrix and thus the cytoskeleton is coupled to the matrix, which allows the cell to perceive how stiff or soft its direct surrounding is. The acquired information will influence the cell morphology and ultimately affect the cell differentiation [2] [3]. Evidently, if the substrate has a higher stiffness than the cytoskeleton of a cell, the cell will flatten and spread across the substrate, while a softer substrate will encourage the cell to retract and assume a rounded shape [2].

Similar to what has been stated for the biochemical environment of an artificial growth matrix, the exact properties will need to be designed according to which cell type the system is targeted at. Intuitively the artificial environment should imitate the native cell environment also in its physical and mechanical properties. Table 4 lists examples of natural tissues and hydrogels used for cell culture, comparing their elastic moduli.

Table 4. *Elastic moduli of different tissues and matrix materials.*

Tissue/Material	**Elastic Modulus [kPa]**	**Source**
Brain tissue	0.1 - 0.5 kPa	[3]
Heart	100 kPa	[43]
Muscle	10 kPa	[3]
Bone	10^5 - 10^6 kPa	[3]
PuraMatrix™ (polypeptide hydrogel)	1.2 kPa	[44]
Matrigel™ (ECM basement membrane)	0.4 kPa	[45]
Poly(ethylene glycol)	2000 - 12 000 kPa	[46]

As another physical aspect for cell culture, it has been widely recognized that a 3D surrounding is needed for cells [5]. This becomes clear when one considers the combination of multiple cells and matrix, rather than single cells, because signaling and adhesion is generated from various points of cell surface [26]. Albeit cells being able to survive and proliferate on 2D substrates, their morphology develops differently in a 3D environment, which ultimately affects their differentiation. For instance, fibroblasts show a flat and spread out morphology with prominent lamellipodia when in 2D culture. In 3D culture however they have been observed to form elongated spindle shapes, but develop no lamellipodia [26]. In order to prove that the observed morphology is brought about by dimensionality, the exact same material of the 3D matrix can flattened to a thin sheet, and

the fibroblasts will acquire a cell shape as was described for the 2D culture [26]. It should be noted that next to 3D matrix systems, there also exist other approaches to create a 3D environment for cells, such as forced floating, hanging-droplet and microfluidic platform methods [5].

Besides stiffness and dimensionality there are, of course, other physical factors that influence the cytocompatibility of the matrix. In the case of 3D hydrogels, the gelation is commonly induced after the cells have been added to the mixture; therefore, care must be taken how the gelation reaction affects the final gel. Likewise, the degradation of the matrix is a crucial factor for the final practicality of the product. The cells will expand and produce their own ECM for support and the degradation profile of the matrix must match this behavior, while retaining the structural support. Finally, the mass transport within the matrix will determine the viability of the cells. Factors including the mesh size of a hydrogel, pore size and charge will regulate the diffusion of nutrients, oxygen and other compounds supplied to the cell, as well as the removal of waste products and toxins. For hydrogels porosity is a function of pore size, density and interconnectivity of the pores [23].

2.3 Functionalization strategies

A great deal of research has been carried out in the field of functionalizing hydrogels to yield better cell response, and many strategies have been explored to bind bioactive compounds into scaffolds. These strategies can be categorized by different means (refer to Figure 4), which will be discussed in more detail in this chapter.

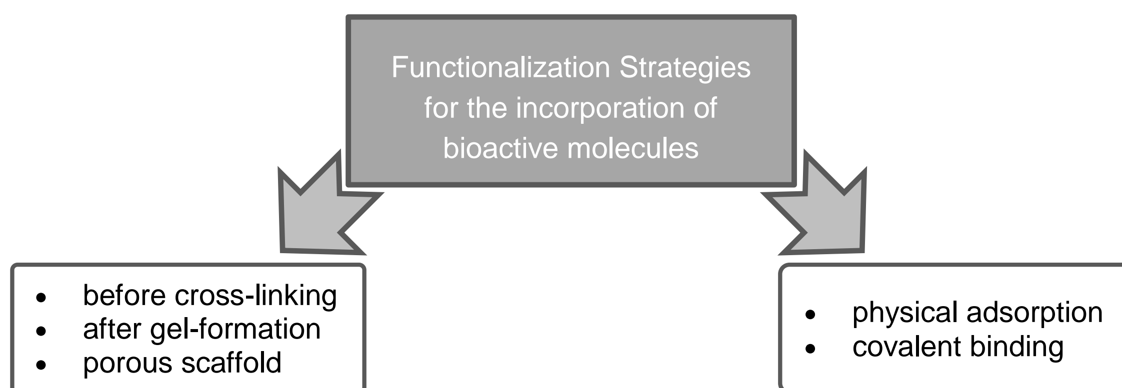

Figure 4. *Categories of functionalization strategies.*

2.3.1 *Physical and covalent coupling strategies*

It is of great importance how the bioactive compound is attached to the hydrogel: Through physical or covalent binding [2]. Whereas the physical binding of compounds is accomplished rather easily, the covalent binding brings stability and specificity.

The bioactive compound can be attached physically to the hydrogel by non-covalent binding, such as electrostatic interaction, van der Waals forces and hydrogen bonding.

This poses a relatively weak binding and consequently, when blending polymer and bioactive compound, it is very likely that the compound will be released quickly upon swelling or implantation of the product [32]. A release of substances may be desired in other application areas, such as drug delivery devices, but is usually avoided for other tissue engineering applications. The strength of physical adsorption can be enhanced by surface treatment of the hydrogel, for example with plasma treatment, which increases the hydrophilicity of the polymer [32]. Another disadvantage for physical entrapment of bioactive compounds in hydrogels is their sensitivity to changes in pH and other properties of the surrounding medium, which again leads to the release of the substances [47].

In contrast to physical binding, covalent coupling largely prevents the release of the bound compounds [47]. On the other hand, finding a suitable method for coupling can be challenging. One requirement of the chemical coupling method is that it must not disturb the structure of the coupled components and also, markedly for avidin, must not alter their functionality. A wide range of different chemical strategies for hydrogels used in tissue engineering have been described in the literature (refer to Appendix A). Here, a few significant strategies will be introduced briefly.

Carbodiimide coupling

A popular method is the carbodiimide coupling with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxyl-succinimide (NHS), which is a technique borrowed from peptide synthesis [48]. Fundamentally a carbonyl group, usually from the polymer scaffold, reacts with the amine group from the bioactive compound. The reaction is facilitated by EDC and NHS forming an intermediate complex (see Fig. 5). There are abundant examples of this method in the literature [8] [10] [31] [42] [47] [49], because it is relatively easy to handle, it is typically a one-step reaction and it is conducted in water and at low temperatures. One crucial aspect appears to be the pH at which reaction is carried out.

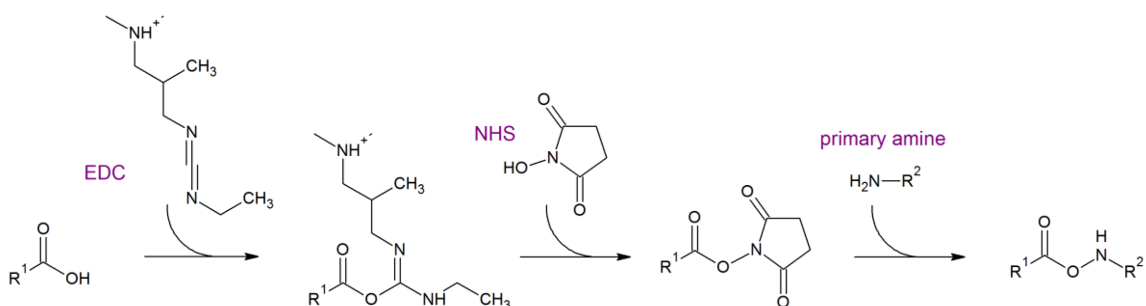

Figure 5. *Carbodiimide coupling strategy with EDC and NHS.*

Employing carbodiimide coupling, Ferris et al. attached an RGD peptide into a purified GG with a conjugation efficiency of 40% [10]. Hobzova et al. demonstrated the covalent attachment of avidin into a poly(HEMA) hydrogel and compare their results to

poly(HEMA) with physically bound avidin [47]. Miyagi et al. succeeded in conjugating the vascular endothelial growth factor (VEGF) with a prefabricated, biodegradable collagen sponge through carbodiimide coupling. The authors point out the immense stability of the covalent binding of the growth factor, with a release of less than 0.08% within three days from the scaffold [49]. In a similar fashion, Reis et al. coupled the peptide sequence QHREDGS onto chitosan and then blended with collagen to form hydrogel, thus improving the survival and maturation of cardiomyocytes in such hydrogels [31]. Zhu et al. report enhanced cell binding ability of PEGDA hydrogels coupled with a cyclic RGD peptide. In their study, the authors describe the formation of a stable intermediate of the NHS-activated PEGDA polymer chain, followed by the coupling with lysine modified peptide sequence [42].

Click-chemistry

Another attractive method of chemical conjugation is the area of click-chemistry, including reactions such as Diels-Alder [4+2] cycloaddition and Huisgen 1,3-dipolar cycloaddition. Clear advantage of these methods is their high specificity, reduced probability of side-reactions [39], and that they are able to be carried out in aqueous environment [41]. Silva et al. demonstrated the use of the [4+2] cycloaddition between a furan-modified GG and a maleimide-modified peptide sequence (GRGDS). Likewise He et al. used the click reaction between azide and propargyl group to graft BMP-peptide to a copolymer of poly(lactide) and poly(ethylene glycol) [39].

Maleimide-thiol coupling

Kubinova et al. attached fibronectin subunits into a poly(HEMA) hydrogel via maleimide-thiol coupling. They exploited the fact that fibronectin exists as a dimer, cross-linked with a disulfide bond. After splitting the dimer under mild conditions into the thiol monomers, they could easily be reacted with the maleimide terminated poly(HEMA) [50].

Oxime formation for end-group modification of polysaccharides

Bondalapati et al. proposed the modification of polysaccharide end-groups, on the example of alginate and dextran. Rather than employing available carbonyl groups, which may obstruct gel-formation as indicated by the authors, the terminal sugar groups of a polymer chain are modified. This strategy preserves the inherent physical properties of the polysaccharide hydrogel, but decreases the maximum possible concentration of coupling partners. If the reaction is catalyzed with aniline, milder reaction conditions can be achieved [13].

The described chemical functionalization strategies do not form a comprehensive list and many more coupling reactions may exist. However, it is considered advantageous if the chosen reaction can be carried out at low temperatures, in aqueous physiological media

and under mild conditions. Additionally, a low-cost functionalization strategy, regarding cost of chemicals, equipment and time, is preferable. It is imperative that the physical properties of the hydrogel are not changed by the functionalization reaction and that the bioactive compound is not perturbed through the attachment itself [32].

2.3.2 *Functionalization of the polymer or final hydrogel*

The presented functionalization strategies can also be categorized by whether the precursor materials or the final gel product are modified. In other words, the functionalization is carried out either before or after the gel-formation. This distinction has a critical effect on the possible application of the developed hydrogel and the cell culture itself [27].

A hydrogel can be modified chemically before the actual gel-formation step is carried out. In order to modify the polymer precursor of a hydrogel, various covalent and physical coupling methods, as described above, can be used. A major advantage for this approach is the possibility of creating 3D cell cultures, by mixing the un-gelled hydrogel and cells in order to encapsulate the cells. On the downside, a modification of the bulk material is more likely to affect the physical properties of the final hydrogel, compared to the modification of the surface would [5] [27].

A synthetic hydrogel may be functionalized during polymerization, comparable to co-polymerization. This is useful to introduce functional groups directly into the main chain of the polymer. Later, these introduced groups can be used for physical adsorption or chemical coupling of bioactive reagents later in the process. However, when co-polymerizing with a functional monomer, changes in the physical properties of the final hydrogel have to be considered. For example poly(HEMA) has been copolymerized with methacrylate (MA) monomers, in order to enable the electrostatic adsorption of avidin to the hydrogel scaffold [47].

Secondly, hydrogel modification may be carried out before or during the cross-linking or gelation of the hydrogel. Typically, the polymer precursor is in solution and available for chemical coupling, before the cross-linking agent is added to the mixture. This strategy has been demonstrated, for example, by Ferris et al. who initially couple an RGD peptide to a purified GG via carbodiimide conjugation and subsequently add calcium chloride in order to induce the gelation [10]. Likewise Zhu et al. first conjugate the cyclic RGD peptide with the hydrogel polymer precursors and afterwards initiate the UV photo cross-linking [42]. Many other research groups carry out the modification directly during cross-linking, meaning that gelation and coupling happens in one step [39] [40]. In this case the fabrication of modified hydrogels is generally carried out in the following fashion: First the polymer is functionalized, i.e. by binding with the bioactive factor, then the functionalized polymer is mixed with the intended cells and finally the gel formation is induced with the required means. This strategy is useful for the encapsulation of cells and 3D cell culture.

If very dense scaffolds are modified after their formation, they can be used for 2D cell culture. Whereas smaller molecules, for example the bioactive compounds used for modification, can diffuse through the hydrogel, cells seeded on top of a hydrogel cannot diffuse and will remain on the surface. Therefore it is possible to functionalize the bulk of a hydrogel even after it has been cross-linked, as shown by He et al [39]. After they cross-linked their hydrogel (poly(lactide-co-ethylene oxide fumarate), PLEOF) and cut it into smaller samples, it was washed and soaked in the reaction mixture for further modification with a BMP-peptide. Nevertheless the cells could be cultured only on top of the hydrogel, although with good results [39]. Additional surface modification strategies that may be mentioned here include techniques like plasma treatment and UV photo grafting. Even though no bioactive molecules are introduced with these techniques, the surface activation can improve the cell response by means of physical adsorption [32].

A special case that should be discussed when distinguishing surface and bulk modification is the modification of porous scaffolds and hydrogels. Because contrary to the previously stated, the porous scaffolds can be functionalized after cross-linking and additionally allow for 3D cell culturing. In gels with multimodal pore sizes, the smaller pores (nanometer) facilitate the transport of smaller molecules, while the larger pores (micrometer) allow the ingrowth of cells and blood vessels [51]. To illustrate this statement, Kubinova et al. synthesized super-porous poly(HEMA-AEMA) hydrogels and later functionalized these with fibronectin subunits and a laminin-derived peptide. The authors report that a pore size within 10-100 µm is suitable to support the growth of cells [50]. Likewise, Miyagi et al. used prefabricated porous collagen scaffolds to examine their use as cardiac patch when cultured with cells. The authors stated that neither the functionalization with the growth factor VEGF, nor the involved chemical process, significantly altered the structure or the physical properties of the scaffold [49]. Moreover it can be conceived that super-pores allow the ingrowth of cells and consequently hydrogels may not need to be seeded before implantation, but rather allow for the ingrowth of body-own cells [50].

2.3.3 *Characterization of functionalized hydrogels*

Characterization of the produced hydrogel is arguably the most important subject for the functionalization process. On one hand the success of the modification strategy has to be verified. Relevant are the nature of the formed attachment site, e.g. covalent or physical entanglement of the compound in the hydrogel network, as well the functionality of the attached compound. On the other hand, a careful choice of characterization methods is necessary in order to determine whether the properties of the hydrogel are suitable for the intended application. For hydrogels with the scope of medical applications, the properties of interest include: Chemical composition and corresponding biological effect, kinetics, physical properties, as well as mass transport properties [23].

In which manner the protein (avidin) is bound in the hydrogel is of great interest. It is of great consequence whether the protein is merely embedded by physical interaction within the polymer network, or whether it is covalently coupled. A range of characterization methods for protein detection was considered, including fluorescence spectroscopy, X-ray photoelectron spectroscopy (XPS), fluorescence recovery after photo-bleaching (FRAP), electrophoresis separation (SDS-PAGE) as well as enzyme-linked immunosorbent assay (ELISA) [52]. XPS is a surface sensitive technique which measures the emission of photoelectrons after the material's surface is irradiated with x-rays. The resulting spectrum is specific towards elements and chemical bonds in the material and statements of the newly formed bond could be derived [53]. FRAP images the lateral diffusion within a fluorescent-labeled sample, after a defined region has been bleached by a light source. The dissipation of the bleached spot depends on the mobility of the fluorescence label and whether it is tightly bound in one place or can diffuse through the material [54]. ELISA is a technique frequently used in biochemical industry and exploits the highly specific binding between antibodies and antigens. An additional enzymatic substrate produces an optic signal which quantifies the amount of bound antigen [55].

Gel electrophoresis (poly(acrylamide) gel electrophoresis, PAGE) is frequently used in biochemistry for the separation of macromolecules according to their electrophoretic mobility, determined by the charge, conformation and size of the macromolecule. Often proteins are denaturated in order to linearize the protein chains and thus achieve separation according to the length of the protein chain, while effects of protein folding, i.e. the secondary and tertiary protein structure, are eliminated. The samples are deposited onto a poly(acrylamide) gel and subjected to an electrical field. Under denaturating conditions sodium-dodecyl-sulfate (SDS) is commonly used as running buffer [56]. Fluorescent staining of the gel after electrophoresis allows for the use of a lower solution- and protein concentration [57].

Fluorescence spectroscopy can be used to determine the presence of avidin in a formulation. However, it cannot resolve whether the avidin is attached to another structure. Fluorescence spectroscopy relies on the electronic excitation of a fluorophore, which then rapidly emits the absorbed energy as radiation. This luminescence is observed at slightly lower energy, or longer wavelengths respectively, and its position and intensity gives information about conditions of the fluorophore [58].

Another aspect of the chemical structure of a hydrogel is the provoked biological effect, implying biocompatibility, cell response and toxicity. The straightforward method to test the biocompatibility of a material is *in vitro* cell culture. Pure GG hydrogels have already been proven to be biocompatible. Various cell types, including for example rat bone marrow cells [12], have shown good viability cultured in GG hydrogels [12] [59].

The kinetic parameters of a hydrogel include gelation time, swelling rate and degradation profile [23]. For this thesis, however, only the gelation time of the GG hydrogels are of

interest and are therefore assessed qualitatively. In an expanded scope the gelation characteristics of GG are also crucial for the production and possible *in vivo* applications of hydrogels. For *in vivo* applications and for regenerative medicine [23] [51], the time frame of gelation needs to be suitable and reliable. For example if the hydrogel serves as an injectable scaffold for a cell transplant to fill damaged tissue, the gel formation needs to occur locally at the site of the damaged tissue [27]. If the gelation is too fast, the construct will not be transplantable by non-invasive means, while too slow gelation will give poor support to the cells during the initial transplant phase. The *in vitro* gelation time is estimated by tilting the reaction vessel after the addition of a cross-linking agent and observing the time it requires until the hydrogel is not flowing any longer, but retains its shape [23].

The mechanical properties of a hydrogel are an important indicator for the structure and ultimate usability of the hydrogel. It has been well established that mechanical cues, such as rigidity and tensile strength, are important factors of a cell matrix which guide cell regulation and proliferation [2]. In general, the modulus and stiffness of an artificial cell matrix should closely resemble the values of the corresponding natural tissue. For example bone marrow cells need relatively hard and stiff microenvironment, whereas neural cells need comparably softer environment [2]. Throughout the literature many different mechanical testing techniques and approaches have been carried out for hydrogels. However, there are no standardized methods or consistently reported parameters, which obstructs the comparison between reported results of different research groups [60].

There are three main approaches to determine the mechanical properties of sufficiently strong hydrogels: indentation testing, rheological assessment and compression testing. Indentation testing is a relatively easy and straightforward method, where a probe is pressed into the surface of a gel sample, while the required force and moved distance into the sample are measured [23]. Rheology determines the deformation behavior of viscoelastic materials, which show combined behavior of liquids (viscous) and solid (elastic) materials. In a typical experimental setup, the gel sample rests between two parallel plates and torsional shear force is applied. Different moduli can be derived from the variation of amplitude and frequency of the applied shear force. The complex shear modulus G* is a measure of the rigidity of the material and its resistance to deformation. The storage modulus G' reflects the elastic behavior, while the loss modulus G'' reflects the viscous behavior for the viscoelastic hydrogel. The relation of these parameters is given in equation 1.

$$|\boldsymbol{G}^*| = \sqrt[2]{\boldsymbol{G}'^2 + \boldsymbol{G}''^2} \qquad (1)$$

The ratio between the two moduli G' and G'' is referred to as the viscoelastic loss factor tan δ (see equation 2). Interestingly, tan delta can be used as an indicator of network formation in a hydrogel [23].

$$\boldsymbol{tan\,\delta = \frac{G''}{G'}} \tag{2}$$

The compression modulus of hydrogels is determined through compression testing. The gel sample is placed between two measuring heads and the force required to compress the sample for a specific distance is measured. From the sample geometry and applied strain, the resulting stress σ in the material can be determined according to equation 3. The strain ε is the degree of compression of the original sample length, according to equation 4, and is usually given in percentage.

$$\boldsymbol{\sigma = \frac{F}{A}} \tag{3}$$

$$\boldsymbol{\varepsilon = \frac{\Delta l}{l_0}} \tag{4}$$

The obtained stress value σ is plotted against strain ε, to yield the characteristic stress-strain curve, from which the compression modulus, fracture strength and -strain can be extrapolated. It is important to note that the deformation of hydrogels under compressive stress is dependent on the velocity of compression. Whereas under rapid compression sample fracture will occur, under exceedingly slow deformation water will elude from the network and the hydrogel will be left as a flat disk. This behavior was described by Nakamura et al. and attributed to collective network diffusion [61].

Determination of mechanical properties of hydrogels can be difficult, because they may be too weak for conventional testing methods. For instance so-termed 'weak gels' [7] that have no rigorous network structure cannot usually support their own weight, show little to no sample shape and thus carrying out compression testing is not possible. Weak gels are however not entirely liquid, but can retain their shape when tilted in the "tube-tilt test". For these gels other testing methods have to be sought, for example ultrasonic testing such as sonoelastography [23].

Finally, an important characteristic of hydrogels is their ability to allow diffusion and transport within the network, as is required to mimic natural tissue in order to supply gases and nutrients to the cells and to remove waste products [23] [27]. Most important parameter for mass transport is the porosity of the hydrogel, defined by the pore size, amount of pores, distribution, and interconnectivity of the pores. Since hydrogels are in essence hydrated polymer scaffolds, the smallest pore size is determined by the average mesh size of the scaffold. If a compound is able to freely diffuse within the hydrogel is dependent on its size and charge. The transport of small molecules necessary for cell viability, like oxygen and vitamins, is regulated through diffusion, although applied flow will enhance the transport. Larger species like proteins or even cells need pores within the hydrogel in order to migrate into the network. Conclusively the mass transport characteristics of a hydrogel depend both on the hydrogel properties as well as the transported species [23].

All of the introduced methods are well established, however they may have to be adapted and modified to be applied to hydrogels. For instance, the compression testing of hydrogels will have to be carried out in a different manner than for tougher materials. Another problem is posed by the lack of established standards for the testing and characterization methods of hydrogels. This makes the comparison of properties within the results of different sources difficult.

2.4 Gellan gum functionalized with avidin

The approach presented in this thesis is to functionalize the hydrogel gellan gum by covalently binding the protein avidin into the polymer network. Avidin then serves as a coupling point onto which biotinylated compounds can be immobilized. Avidin is a tetrameric protein, which exhibits high specificity and affinity for binding the molecule biotin, also known as vitamin H [62]. Many different biotinylated species, i.e. compounds attached to biotin, including fluorescence markers and peptide sequences, are available commercially. These biotinylated compounds will consequently define the biochemical environment within the hydrogel and guide the cell fate or induce specific cell functions.

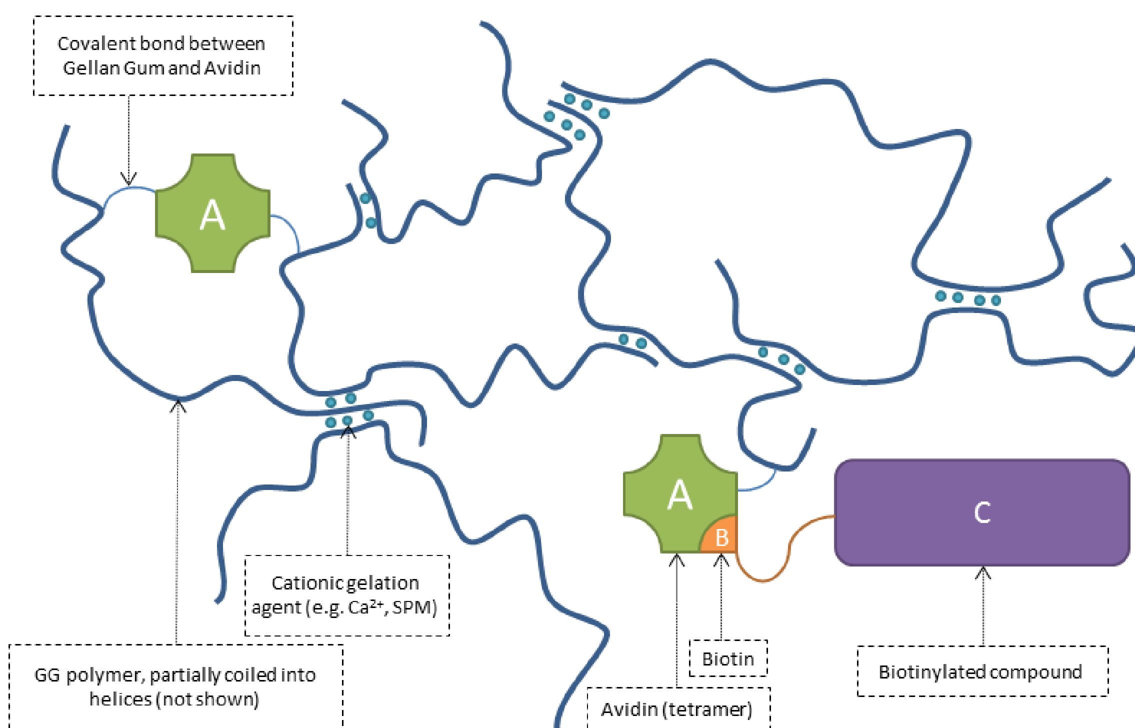

Figure 6. *Schematic representation of gellan gum network with covalently bound avidin. The exemplary biotinylated compound "C" is coupled to the network over the avidin-biotin binding.*

Ultimately, this concept offers a highly versatile hydrogel for cell environment: The mechanical properties of gellan gum can be adjusted by choice and concentration of the gelation agent [7], while the biochemical environment can be adjusted to support a specific cell type by adding a suitable biotinylated cell cue [4] [32]. Figure 6 shows a schematic representation of the described gellan gum network. First the gellan gum

polymer (dark blue lines) will be functionalized with Avidin (green “A”) by forming a covalent peptide bond (light blue line). Right before the gelation agent (small turquoise circles) is added, the gellan gum can be mixed with the desired biotinylated compound (purple square “C” with orange tail “B”). Of course it can be considered to add the biotinylated compound at a different step, for example after gel formation. This would, however, rely on the diffusion of the compound and ultimately its size and charge.

With the addition of a desired biotinylated species during gel formation, motifs can be permanently introduced to the matrix architecture which can mediate cell adhesion and differentiation [2]. Anchoring these cues gives a stark contrast, as these cues were traditionally added to the cell culture medium as soluble compounds [4], which however will eventually diffuse out of the system and not be available for the cells. Furthermore, it is intended so, that the avidin-functionalized hydrogel is produced and stored, without biotin-binding sites being taken up. Thus the actual cell-specific modification can be carried directly before the hydrogel is seeded with cells. Additionally, a mixture of different biotinylated species can be used and thus a combination of different cues is made available to the cells (refer to 2.4.1 and Fig. 7).

The challenges for this approach mainly relate to the preservation of the originally determined properties of GG and nChiAvd. The gelation and final gels should not be affected by the introduced functionalization, nor should the addition of the biotinylated compound interfere with the gel formation. Furthermore, other properties, such as the transparency of the final gel, need to be retained if it is intended to use in cell modeling applications. Similarly, the deployed avidin must retain its structure and functionality. Therefore, the chemical strategy needs to be mild and the used solvents suitable.

2.4.1 *Avidin and the avidin-biotin binding*

Avidin is a glycoprotein that consists of four identical subunits and is derived from egg white [63]. The ribbon structure of avidin is shown in Fig. 7 A. Each of these subunits possesses one binding pocket for biotin, also known under the name vitamin H and coenzyme R (Fig. 7 B). The interaction between avidin and biotin is to be one of the strongest non-covalent bonds that have been discovered [64]. The high affinity and specificity between the two compounds has led to frequent utilization of the system for bioconjugate techniques [63]. Derivatization of the valeric acid side chain of biotin (highlighted in purple in Fig. 7 B), i.e. the carboxyl terminus, is a convenient tool for coupling reactions of biotin to other compounds, for example peptide sequences, whole proteins, antibodies, fluorescence markers, nucleic acids or other markers [65]. Biotinylation reactions are not a topic within this thesis, because a wide variety of biotinylated species, including those used in the experimental part, are available commercially.

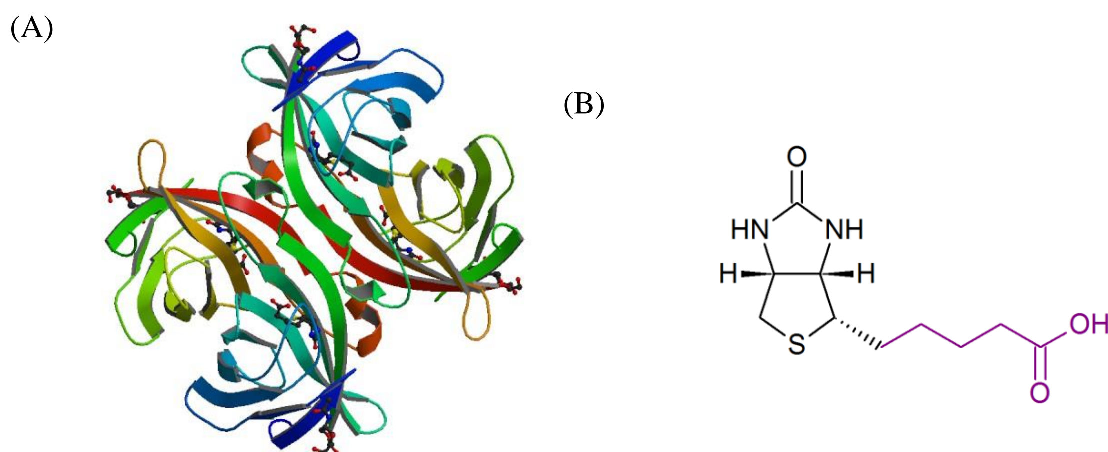

Figure 7. *Avidin structure as ribbon diagram [66](A) and the chemical structure of biotin (B).*

An outstanding aspect of avidin is its relatively high stability towards extreme conditions, including elevated temperature, low pH and denaturation agents such as urea and guanidine hydrochloride [63]. Even under these conditions avidin is able to retain its structural integrity and activity. Moreover, if biotin is bound by avidin, the complex exhibits even higher stability. Breaking the biotin-avidin bond requires a pH of 1.5 and high concentrations of guanidine hydrochloride. Large compounds coupled to biotin are, however, expected to diminish the binding strength between avidin and biotin [63].

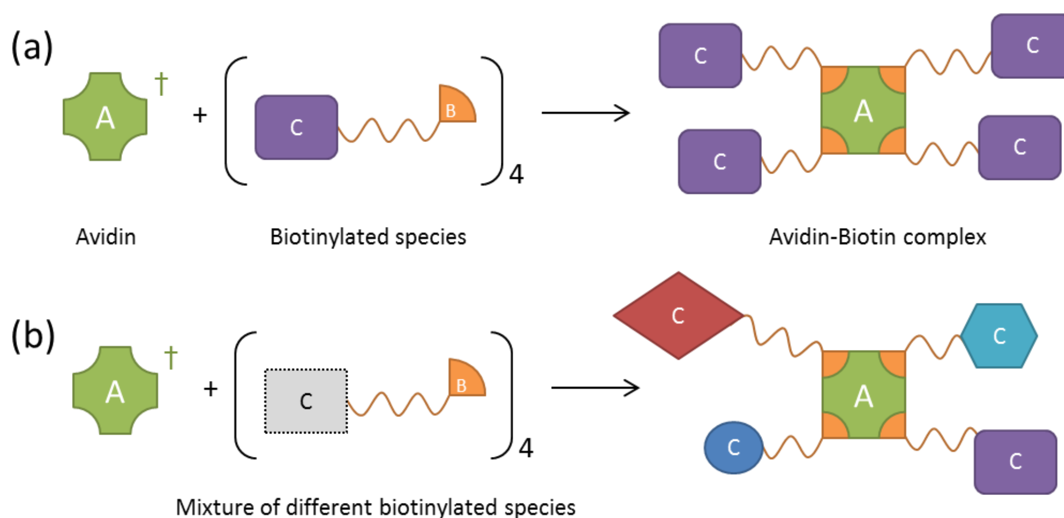

Figure 8. *Schematic of avidin-biotin binding with a single type (a) and a mixture (b) of biotinylated species.*

Fig. 8 schematically shows the formation of the avidin-biotin complex. In the upper part (a) a single type of biotinylated compound is used, whereas in the lower part (b) a mixture of different biotinylated species is bound by one avidin tetramer [47] [63]. It is widely accepted that for a successful cell modelling multiple cues have to be made available to the cells. Functionalizing the hydrogel matrix with avidin and supplying a mixture of biotinylated cell cues will be able to serve this requirement.

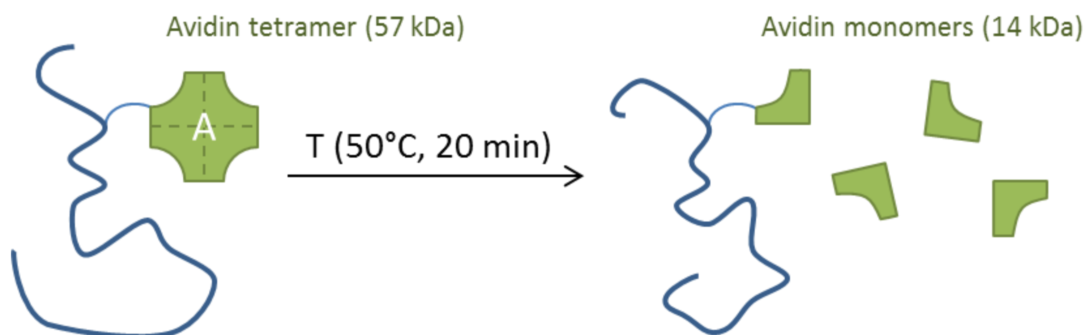

Figure 9. *Schematic representation of avidin tetramer separation into monomers [67], while one of the monomer blocks is coupled to the gellan gum chain (blue line).*

If avidin does face destabilizing conditions, then the tetramer will split into its monomers. Fig. 9 shows the cleavage of avidin monomers schematically [67]. As mentioned earlier, avidin is a tetramer consisting of four identical monomer. Protein folding and adhesion of subunits relies on hydrogen bridge bonding. When the native protein folding is disrupted and these weak bonds are interrupted, the structure and functionality of the protein is abrogated [68].

Avidin is a rather alkaline protein with an isoelectric point (pI) of around 10, which may present a disadvantage in certain cases. The positive charge on the surface of the protein can lead to ionic interaction with negatively charged species [63], for example cells or polysaccharides, e.g. gellan gum. To circumvent the disadvantages of avidin, the bacterial analogue streptavidin can be used. Streptavidin is functionally similar to avidin, has the same high affinity and specificity to biotin, but has a much lower pI of around 5-6, due to considerable differences in the primary protein structure [63]. However, the production of streptavidin is more intricate and the product is magnitudes more expensive.

For this thesis project a modified avidin version, a neutralized chimeric avidin (nChiAvd) was used, as described by Hytönen et al. [69] and Ray et al. [68]. The Protein Dynamics group of docent Hytönen first produced a chimeric avidin by replacing a specific sequence in chicken avidin with a sequence from the avidin related gene number 4 (AVR4), as well as introducing a point mutation (I117Y). The produced protein ChiAvd(I117Y) was reported to show an even higher thermal stability of up to 110°C than the natural avidin. Natural avidin does have the higher affinity towards biotin, with a dissociation constant of 3.7 $x10^{-4}$ s^{-1}, compared to the dissociation constant of 1.2 $x10^{-3}$ s^{-1} for the chimeric avidin. This value is nevertheless exceptionally high and with its enhanced stability ChiAvd is an outstanding candidate for bioconjugate systems. Further the ChiAvd is charged neutralized by introducing several mutations in the protein chain. The pI of the charge neutralized chimeric avidin (nChiAvd) is lowered to 6.9, which is expected to avoid any interaction with negatively charged compounds, such as gellan gum. The neutralization does not affect the thermal stability or affinity constant of chimeric avidin [69].

3. EXPERIMENTAL PART

3.1 Sample preparation

For all sample preparation and characterization techniques, de-ionized (DI) water was used. Most of the chemicals were obtained from Sigma-Aldrich, unless specifically stated otherwise. The neutralized chimeric avidin (nChiAvd) was a kind gift from the Protein Dynamics research group of BioMediTech, UTA. Other materials and chemicals that were used are mentioned in their respective point and listed in Appendix B. The chemical functionalization and gelation experiments were carried out in the Biomaterials and Tissue Engineering laboratory in the department of Electronics and Communication Engineering.

The protocols for purification and gel sample preparation are not, in essence, further modified from their respective original protocols. For the functionalization, however, several details of the protocol needed to be refined for the coupling with avidin and a range of different conditions, especially solvents, were investigated. Step-by-step protocols for the purification and functionalization are given in appendix E and F.

3.1.1 *Purification of gellan gum*

Small batches of gellan gum (Gelzan^{TM}, Sigma Aldrich) were purified at a time in order to remove divalent cations, mainly calcium, according to the method established by Doner et al. [19]. Here, 500 mg of GG are dissolved in 100 mL DI water and heated in a water bath at 60°C to give a 0.5% solution of GG (5 mg/mL). After the GG has dissolved, 1.5 g of the Dowex cation exchange resin (H+ form, 50-100 mesh, pre-rinsed in HCl (1 N) and DI water) are added and the mixture is stirred for 30 min in the water bath at 60°C. After the resin settles the clear supernatant is transferred and filtered through a coarse filter (filter paper grade 4). Ideally the filtrate is kept over a waterbath at all times in order to prevent gelation upon cooldown. Sodium hydroxide solution (NaOH, 1 M) is added dropwise to the filtrate and the pH of the solution is followed by testing with pH paper. After a pH of 7.5 is achieved, the solution is stirred for further 5 min. Finally, the now sodium-purified GG (NaGG) is precipitated by pouring the warm solution into two beakers with 100 mL isopropanol each. The precipitate is dried first by vacuum filtration and subsequently by lyophilization over three days.

To verify the success of the purification, the produced NaGG was analyzed by flame atomic absorption spectroscopy (AAS), as described in chapter 3.2.1.

3.1.2 *Functionalization of sodium purified gellan gum*

The sodium purified GG (NaGG) is covalently coupled with the protein avidin using carbodiimide chemistry. The reaction conditions are very mild and unused reactants or

waste products, which are potentially detrimental for later application, are removed by dialysis. The protocol for activation of NaGG is based on the publication by Ferris et al [10]. First, 100 mg of lyophilized NaGG (1.0% w/v, 10 mg/mL) are chopped into smaller pieces and dissolved in 10 mL HEPES buffer ([2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid], 50 mM, pH 7.5) in a water bath at 60°C and stirred with a magnet-stirrer. Upon dissolution the water bath temperature is decreased to 40°C, and EDC (0.3 M, 50 µl, in HEPES buffer) and NHS (0.15 M, 50 µl, in HEPES buffer) are added to the solution. The activation is carried out for 15 min at 40°C, after which it is quenched with β-mercaptoethanol (14 µl, final concentration 20 mM). Subsequently avidin (CNCA, 45 µl, 3.540 mg/mL) is added to the reaction mixture and the solution is stirred for 5 h in a water bath at 40°C. The product is then dialysed against DI water over five days, followed by lyophilization over two days.

The solid products (NaGG and NaGG-avidin) are stored in a dry place, or in the refrigerator at +4°C. Once dissolved, the solutions are expected to be stable for one month.

3.1.3 *Gel sample preparation*

The GG sample is dissolved in a 10% sucrose solution of either water (pH 5.6) or HEPES buffer (50 mM, pH 5.5…7.5), to yield a 0.5% w/v (5 mg/mL) solution. Higher concentrations were tried out for NaGG-avidin samples, reaching up to 0.7% w/v solutions. The warm solutions (50°C) were pressed through a syringe filter (0.2 µm) for sterilization. The cross-linker solutions spermine (SPM), spermidine (SPD) and calcium chloride ($CaCl_2$) are prepared in a 10% sucrose solution and sterile filtered through a syringe filter (0.2 µm). Before the gels are prepared all solutions are warmed in a water bath at 37°C. The gels are prepared in a round plastic mold with the dimensions of 1.2x 0.6 cm. The gel samples are prepared so that 80 µl of cross-linker solution are pipetted into the well and immediately 500 µL of the GG solution are added. It is important to note that although concentrations, solvents and species are varied, the volume ratio of 25:4 polymer-to-cross-linker solution ratio is kept constant. The hydrogel sample is produced by pipetting the polymer and cross-linker solutions into a plastic mold and mixing is achieved through repeated pipetting. Care must be taken that no air bubbles are introduced into the gel while pipetting. The gel samples are covered to prevent them from drying out and stored at 4°C overnight before the gel samples are analyzed and compression tested.

3.2 Characterization methods

In the following chapter the methods employed to characterize the prepared samples are described. The elemental analysis with AAS and compression testing were carried out in the department of Electronics and Communication Engineering, as well as in the Department of Chemistry and Bioengineering in TUT. The fluorescence spectroscopy

and the gel electrophoresis were carried out in the BioMediTech facilities in FinnMedi, UTA.

3.2.1 *Atomic absorption spectroscopy*

Elemental analysis for calcium and sodium was conducted using flame-atomization atomic absorption spectroscopy (AAS, Perkin Elmer, AAnalyst 300 Atomic Absorption Spectrometer) with the hollow cathode lamp light sources (Perkin Elmer, Lumina^{TM} lamps) for calcium (422.7 nm, linear range 5.0 mg/L) and sodium (589.0 nm, linear range 1.0 mg/L) and an air/acetylene oxidant/fuel mixture. The stock standard solutions and their dilutions were prepared according to Perkin Elmer instructions [70], and all glassware was rigorously cleaned beforehand. For the calcium standard, calcium carbonate ($CaCO_3$, 312 mg, 500 mg/L final concentration) in 50 mL DI water were dissolved with the dropwise addition of about 6 mL hydrochloric acid (HCl, 1N) and diluted to 250 mL with DI water. The calibration standards were diluted to 0.5, 1.0, 1.5, 2.5 and 5.0 mg/L. For the sodium standard, sodium chloride (NaCl, 636 mg, 1000 mg/L final concentration) was dissolved in 250 mL DI water. The calibration standards were diluted to 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L. The GG and NaGG sample was digested prior to characterization following the protocol from Kirchmajer et al. [20]. A small amount of sample (200 mg) was dissolved with concentrated sulfuric acid (H_2SO_4, 1 mL, 98%) and lightly heated until the solution was dark brown and homogeneous. Hydrogen peroxide (H_2O_2, technical grade, around 5 mL) was added dropwise until the solution turned clear, but retained a yellow color. After the solution cooled to room temperature, a potassium chloride solution (KCl, 3.2 mL, 0.07 M) was added as ionization suppressant. The mixture was diluted to 20 mL with DI water and measured within 24 h [20].

3.2.2 *Fluorescence spectroscopy*

Fluorescence spectra of the non-gelated samples were recorded with the QuantaMaster™ spectrofluorometer (Photon Technology International, Inc., Lawrenceville, NJ, USA). For analysis the biotinylated fluorescence dye biotin-5-fluorescein (B5f) was used, which has an excitation point of 490 nm and an emission peak in the range of 500 to 600 nm. When B5f is coupled to avidin, via the biotin-avidin binding, the fluorescence capacity is quenched by roughly 50% [71]. Background spectra of the used solvents, namely 10% sucrose and HEPES buffer, were recorded, but no fluorescence in the emission range was observed.

Fluorescence spectra were taken from 0.1% w/w (1 mg/mL) samples of NaGG-avidin and NaGG physically mixed with nChiAvd (1.6 µg of nChiAvd per 1 mg NaGG). To fill the cuvette 2 mL of solution were required. The samples were analyzed without any fluorescence dye, with added B5f (10 µL, 20 µM, 5 min incubation), and with added biotin (14.4 µL, 645 $\times 10^{-4}$ M, 15 min incubation) and subsequently B5f (10 µL, 20 µM, 5 min incubation).

3.2.3 *Gel electrophoresis*

To carry out gel electrophoresis (SDS-PAGE), NaGG-avidin and NaGG+avidin samples (100 µL, 0.5% w/w, in HEPES 50 mM pH 7.5) were combined with 25 µL loading buffer, and biotin (1.6 µL, 85 µg/mL) was added to the biotin-samples. Avidin (nChiAvd, 7.91 µg/mL, in DI water) solution was prepared as a control sample. All solutions were incubated at 50°C for 20 min. The samples (sample volume 10 µL) were loaded onto a pre-cast gel (Mini-PROTEAN® TGX™, BioRad) and only then the running buffer was added to the tank and the fluid level was carefully raised above the wells, so that the deposited samples would remain in their place. The electrophoresis was carried out for 30 min at 200 V. Afterwards the gel was stained with Oriole™ Fluorescent Gel Stain [57] solution for 90 min and subsequently flushed three times with water to remove dirt and dust before imaging.

3.2.4 *Compression testing*

The gel samples are subjected to compression testing in order to evaluate their mechanical properties and determine their suitability as growth matrix for specific cell types. Gel samples are prepared according to chapter 3.2.3, which are tested to their compression behavior with a Bose BioDynamic ElectroForce Instrument 5100. The Bose instrument offers the "tuneIQ" program which finely tunes the apparatus to the toughness of the sample, in order to achieve the most accurate response.

The compression test is carried out at ambient pressure and temperature and before the compression was carried out the height and width of each sample was recorded. The instrument has two opposite metal heads, so-called platens or pistons, which are both wrapped in parafilm and covered with wetted cellulose paper in order to prevent the sliding of the gel sample. The upper platen is connected to electrically controlled pressure sensor, while the bottom platen is moving to the desired displacement value. Figure 10 shows the schematic measuring set-up. The gel sample is carefully placed onto the bottom platen, manually moved into contact with the upper platen and the compression was carried out with a compression speed of 10 mm/min to 65% of their original height. To calculate the compression response, the displacement, area onto which the force was applied, and the measured load are recorded.

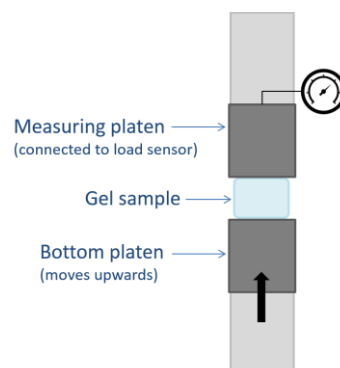

Figure 10. *Measuring set-up for compression testing with the Bose instrument.*

4. RESULTS AND DISCUSSION

In the following chapter the results of the purification and functionalization of gellan gum will be presented and discussed accordingly. Firstly, a couple of fundamental parameters for the initial experimental set-up will be discussed, such as the purification of gellan gum and the medium of the functionalization, along with a brief discussion of the reaction mechanism. Next, the outcome of the functionalization will be analyzed from the fluorescence spectra and gel electrophoresis. Finally, the ability of the modified gellan gum to form true gels will be examined on the basis of the prepared gel samples, as well their behavior under compression.

4.1 Initial considerations

The purification of gellan gum was carried out in order to remove the combination of mono- and divalent cations present in the commercial product, and replace them with sodium ions. As reported by Ferris et al. the purification also increases the carbodiimide conjugation efficiency and prevents premature gelation upon cooldown [10]. Furthermore, it can be assumed to be beneficial for a controlled cell culture to reduce the excess amount of calcium. Elemental analysis was carried out for pure gellan gum and for the purified sample. The results are shown together with comparison values from the literature in Table 5.

Table 5. *Results of the elemental analysis for Na^+ and Ca^{2+} on GG and NaGG, as well as a comparison to literature values [10], [20].*

	Na^+ (w/w)	Ca^{2+} (w/w)	Source
GG	*0.60 %*	*1.20 %*	*Ferris et al. (2015)*
	0.55 %	*1.36 %*	*Kirchmajer et al. (2014)*
	1.30 %	0.15 %	own result
NaGG	*2.50 %*	*0.06 %*	*Ferris et al. (2015)*
	2.51 %	*0.04 %*	*Kirchmajer et al. (2014)*
	2.75 %	0.04 %	own result

The purification does indeed cut the calcium concentration and doubles the amount of sodium present in the gellan gum. If the obtained values are compared to literature values [10], [20] however, large discrepancies are apparent. The concentration of sodium in the unpurified GG is twice as high, whereas the calcium concentration is significantly lower compared to the values from Ferris et al. and Kirchmajer et al. Contrarily the obtained values for the purified NaGG match the literature values rather closely. Currently it is unclear, to what this considerable discrepancy is due, if a high batch-to-batch

homogeneity is assumed. The raw gellan gum was the same type and obtained from the same source and producer as Ferris et al. and Kirchmajer et al. did: Gelzan^{TM} CM from CP Kelco U.S., Inc. The measured calcium concentration for GG was slightly out of the linear range with 7.5 mg/L, which may give a somewhat inaccurate value, but cannot, however, explain this large deviation from the literature values.

A suitable solvent had to be attained for carrying out the activation reaction and subsequent Coupling reaction between gellan gum and nChiAvd. On one hand the activation reaction with EDC and NHS has to be carried in a narrow pH range up to pH 7.2. On the other hand, the stability of nChiAvd and the reactivity of the terminal amines of nChiAvd have to be considered. Usually nChiAvd in solution is stabilized with the addition of sodium chloride. This had to be relinquished however, because an increased amount of cations leads to the gelation of gellan gum, or at least to a highly viscous solution, which is difficult to work with. For reference, appendix C shows a table of NaGG and nChiAvd in different solvents, to give an impression of the limitations in solvents. Here, the activation reaction is carried out in HEPES buffer at pH 7.5, without any addition of sodium chloride.

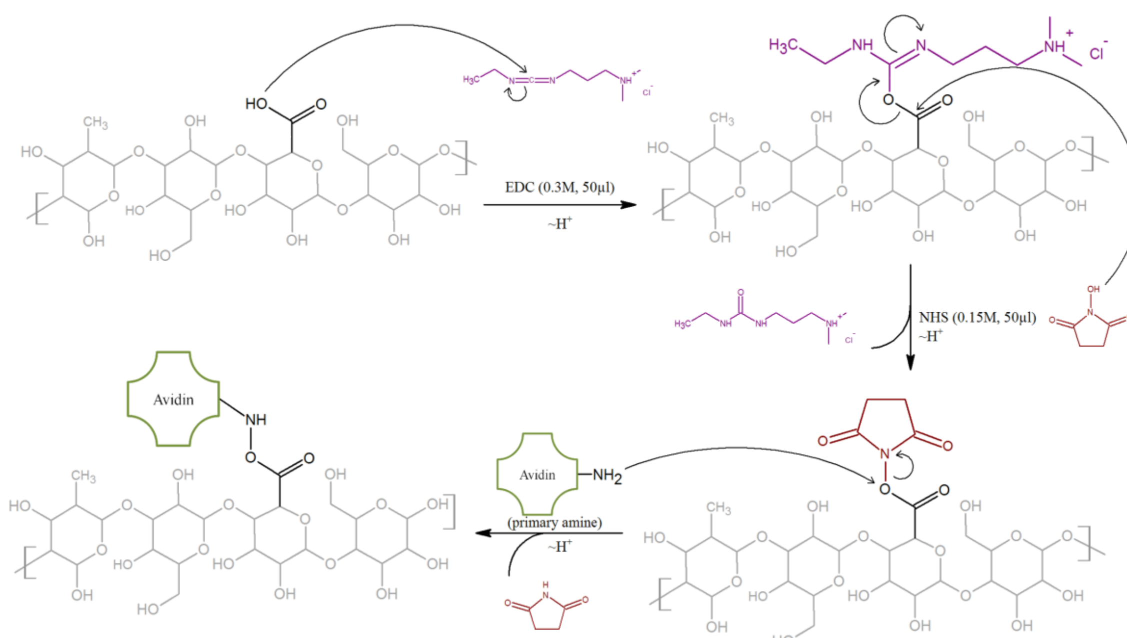

Figure 11. *Chemical mechanism of the activation of GG with EDC and NHS and subsequent functionalization with avidin (primary amine).*

The carbodiimide reaction exploits the presence of the single carboxyl group (-COOH) per repeat unit of the gellan gum polymer, as well as the abundance of amino groups (-NH_2) of the protein nChiAvd. The reaction steps of the activation with EDC and the stabilization of the formed reactive group with NHS is shown in Fig. 11. The carboxyl group readily reacts via nucleophile attachment to the imine group of EDC to form an acylisourea active ester. This isourea intermediate is rather unstable and the addition of NHS will convert it to the amine-reactive NHS ester. The NHS ester is less susceptible to

hydrolysis and readily reacts with the primary amines presented by nChiAvd [48]. The limiting reagent in this process is the nChiAvd, which is calculated to take up 2% of the total amount of carbonyl groups available in the gellan gum.

There are a few side reactions that can be considered for this coupling strategy. Cross-linking may occur between gellan gum chains due to activated carbonyl sites. EDC is a highly reactive and sensitive compound which rapidly deactivates under hydrolysis at air and in aqueous solution. Furthermore EDC shows its highest reaction efficiency at a pH of around 4.5, however the reaction was carried out at a pH 7.5 in order to stabilize the nChiAvd [48]. EDC would also have the ability to activate the carbonyl groups of the nChiAvd and thus couple two proteins together. The quenching step with β-mercaptoethanol, which deactivates all remaining EDC, is carried out before the addition of nChiAvd to prevent this. This quenching step also deactivates all remaining activated sites in the GG.

4.2 Success of functionalization

Theoretically there are three ways nChiAvd can be embedded into the GG network. If there are no bonds or other interaction between the protein and GG, the protein can easily diffuse within and out of the network. Normally the stability and functionality of avidin in solution depends on the medium and salt concentration. In contrast when bound to a network structure it can generally be assumed that nChiAvd will be less susceptible to aggregation, as compared to freely diffusing. Secondly the protein could be physically entrapped into the network by electrostatic interactions. Whereas this may result in a relatively stable attachment, the intention was to avoid this by using a charge neutralized version of avidin. Finally, the strategy at hand is to bind the protein covalently into the network. It will be directly, chemically coupled to the polysaccharide chain at one or more points. However, this strategy can be challenging, because the coupling reaction and the formed bond itself, must not affect the hydrogel structure, the ability to form gels, nor the functionality of the protein.

In order to verify the presence and functionality of nChiAvd in the gellan gum network, fluorescence spectra were recorded. Therefor the biotinylated fluorescence dye biotin-5-fluorescein (B5f) was used, which gives an emission peak around 500-600 nm. The fluorescence intensity of B5f is decreased by roughly 50% if its biotin tail is bound to nChiAvd [71]. Hence the fluorescence molecule is attached to avidin over the avidin-biotin binding. Fig. 12 shows the fluorescence spectra of coupled NaGG and nChiAvd (NaGG-avidin) with added B5f, as well as added biotin and B5f.

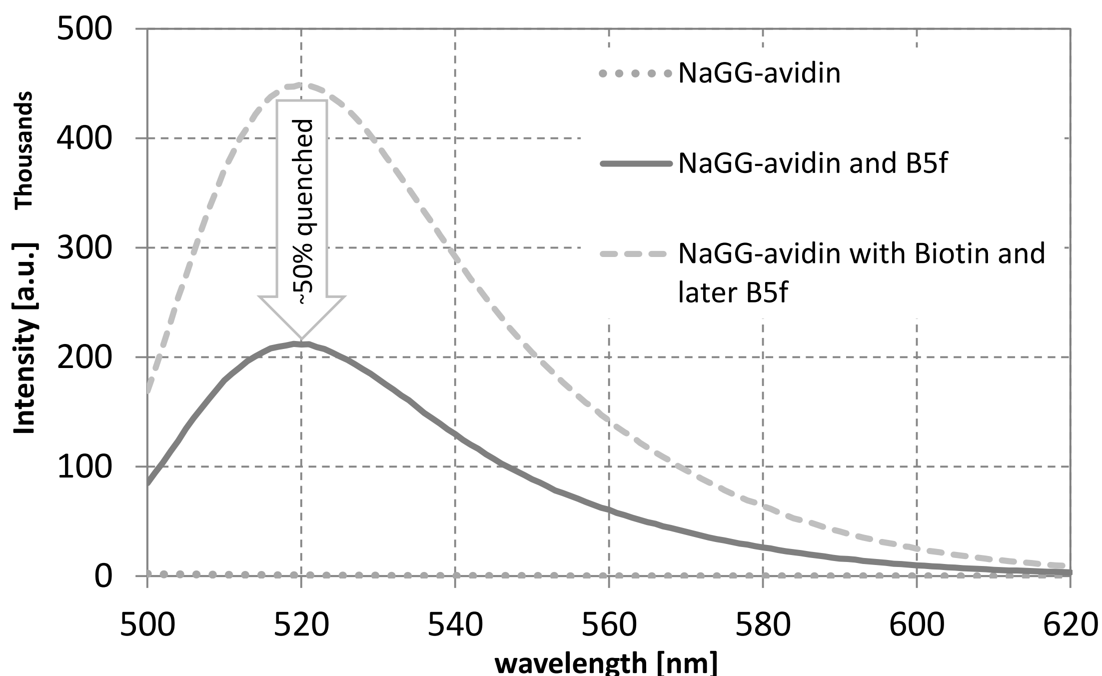

Figure 12. *Fluorescence spectrum of NaGG-avidin. Dotted line: pure NaGG-avidin; Solid line: NaGG-avd and B5f, quenched fluorescence; Dashed line: NaGG-avd with added biotin, before the B5f is added.*

The full fluorescence intensity of B5f is displayed when the biotin binding sites of nChiAvd are already taken up by biotin (see dashed line, Fig 12). Upon binding to nChiAvd, the fluorescence intensity of B5f is clearly diminished (see solid line, Fig. 12). The observed quenching of B5f activity determines the presence of nChiAvd in the system, as well as its functionality and ability to bind biotin. The fluorescence spectrum of pure NaGG-avidin is shown to demonstrate that it exhibits no background fluorescence.

For comparison with the functionalized NaGG-avidin sample, fluorescence spectra of the solvent HEPES, as well as a physical mixture of NaGG and nChiAvd were recorded. An identical amount of B5f was added to each sample before measuring. Figure 13 shows the rather surprising result of the measurement, where the NaGG-avidin sample exhibits higher fluorescence intensity than the pure solvent without any avidin. Also, the physical mixture of avidin and NaGG (NaGG+avidin) exhibits drastically lower fluorescence intensity than the NaGG-avidin sample. Theoretically, it would be expected that the physically mixed (NaGG+avidin) and coupled (NaGG-avidin) exhibit a similar fluorescence intensity, because no difference in binding functionality through chemical coupling is anticipated. Further, the fluorescence spectrum of B5f in HEPES buffer should have higher intensity, compared to avidin containing samples.

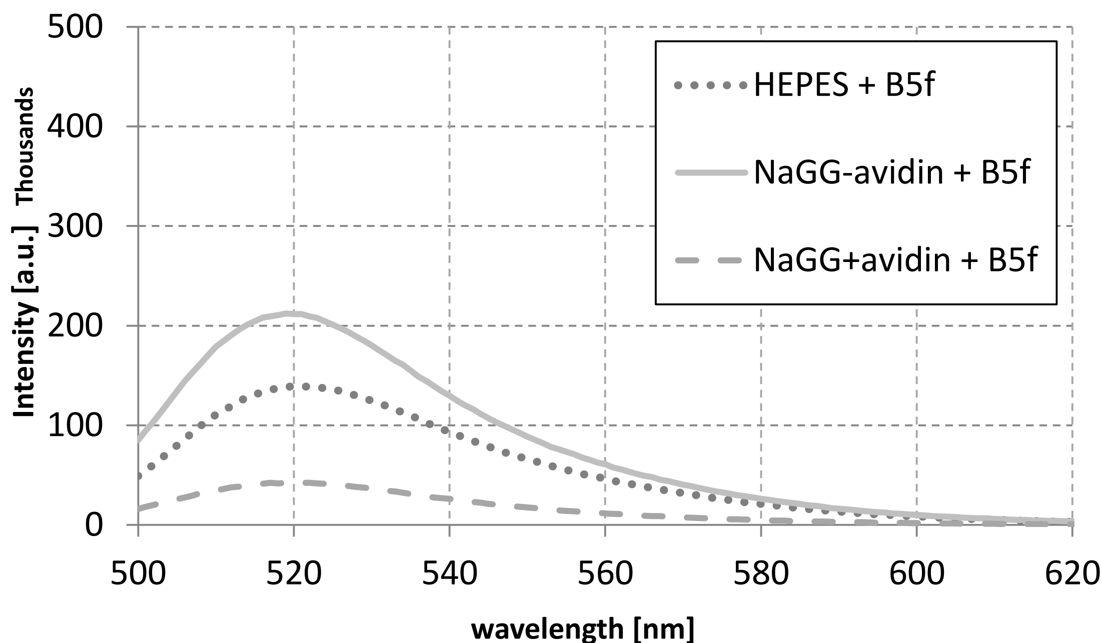

Figure 13. *Fluorescence spectrum of different substances with identical amount of B5f. Dotted line: HEPES (solvent of the other gellan gum samples); Solid line: NaGG-avidin (0.1 wt%, same as shown in Fig. 12); Dashed line: NaGG +avidin (0.1 wt%).*

The decreased fluorescence intensity of NaGG+avidin compared to NaGG-avidin may be explained with a higher amount of active nChiAvd than can be found in the coupled version NaGG-avidin. Although the same amount of nChiAvd was added to both formulations, it is not expected that the all of the nChiAvd is successfully coupled to the activated NaGG. The degree of functionalization and the yield of the coupling reaction were not investigated in more detail. From this spectrum (Fig. 13) it could be concluded that there is little to no functional avidin present in the NaGG-avidin samples. This, however, is refuted by the successful fluorescence quenching shown in Fig. 12. Similarly, it is not currently clear why the NaGG-avidin sample exhibits a more intense fluorescence peak than the fluorescence dye in buffer solution (HEPES + B5f).

Fluorescence analysis cannot however give any evidence towards how the nChiAvd is attached to the gellan gum network. Therefore, gel electrophoresis was carried out to analyze the interaction between nChiAvd and GG. For comparison, a sample where gellan gum and nChiAvd were simply mixed, without any activation or coupling chemistry, was prepared (GG+avidin).

Oriole fluorescent staining technique was chosen, because unstained gels require higher protein concentrations and the high solution viscosity of NaGG-avidin samples proved to be problematic. Fig. 14 shows an image of the oriole-stained SDS-PAGE result. Samples of nChiAvd and nChiAvd-biotin are analyzed as well, to verify the positions of the nChiAvd monomer and tetramer bands. The NaGG-avidin (2) and and GG+avidin (4) show strong bands for monomeric nChiAvd, whereas NaGG-avidin+biotin (3) shows

only a very faint monomer band and GG+avidin+biotin (5) no monomer band is visible. However, an avidin tetramer band is visible for (5). The nChiAvd (6) separates into monomers after the incubation steps, unless it is stabilized with biotin, as seen in for nChiAvd+biotin (7), where both a tetramer band and a faint monomer band are visible.

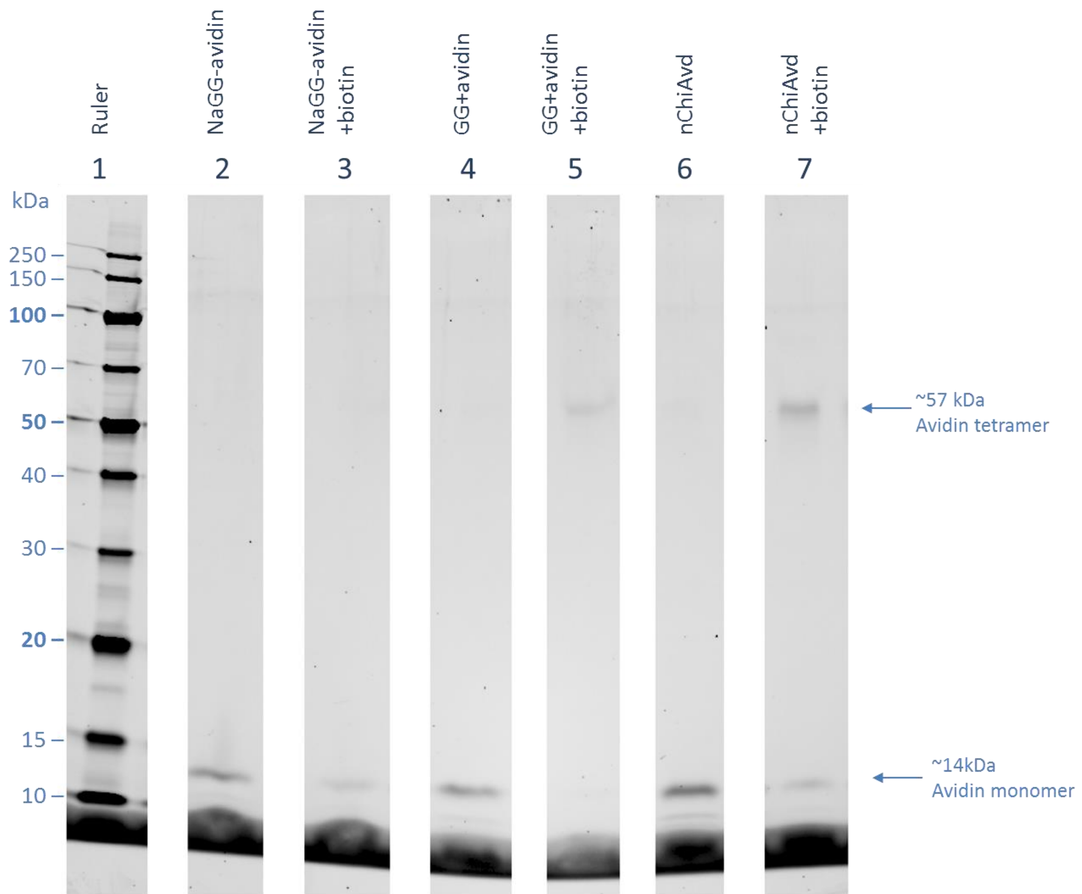

Figure 14. *Image of the SDS-PAGE gel, oriole stained, 200 V for 30 min.*

To make a statement about the binding between gellan gum and nChiAvd, samples (3) and (5) should be carefully evaluated. From the coupled samples NaGG-avidin+biotin (3) no tetrameric nChiAvd is released, in contrast to the physical mixture of GG+avidin+biotin, where a tetramer band is clearly visible. The monomer band in (2), (3) and (4) can be explained with the separation of nChiAvd monomers from the tetramer, because not all monomer blocks of the tetramer are attached to the gellan gum network. As was explained in chapter 2.4.1 and Fig.9, the avidin tetramers can cleave into monomers. This behavior is not observed for (5), and only very faintly for (3), because biotin stabilizes the nChiAvd tetramer [69]. The coupling points between GG and nChiAvd are deliberately created in low amounts, in order to not disturb the protein structure of nChiAvd, as well as maintain a sufficient amount of carboxyl groups for the gelation of gellan gum.

From these results it can be safely argued that the coupling reaction between GG and nChiAvd has been successful and the nChiAvd retains its functionality to bind biotin. For

further optimization of the functionalization reaction it may be beneficial to reconsider the reaction conditions of the functionalization, such as the pH of the solution, in order to optimize the reaction efficiency and yield.

An important point of discussion remains to be the concentration of nChiAvd in relation to gellan gum as well as the nChiAvd density in the final hydrogel. At the moment the deployed amount of nChiAvd relies on the reported values by Ferris et al, who determined 2% of all carboxyl groups in GG to be the optimal amount to be used for coupling with RGD [10]. In order to determine the optimal spatial density of cues coupled to the network, two factors have to be balanced. On one hand the modification of the polymer has to be imperceptible enough for the macromolecular properties of the gel to remain unaltered and the cross-linking sites must not be impaired. Bernstein-Levi et al. report the change of rheological properties in polysaccharide hydrogels when functionalized with two different amounts of RGD. The authors claim that coupling of RGD has a direct effect on the conformational state of the individual polymer chain. A relatively low amount of RGD induced a Newtonian fluid behavior in the non-gelated polymer, whereas a larger amount resulted in increased shear-thinning [72]. On the other hand, the spatial density of cues in the network is anticipated to be crucial for cell recognition and integrin binding. On 2D substrates the ligand spacing has been shown to have tremendous effects on cell adhesion and differentiation of anchorage dependent cell types [29]. For the future development of the produced NaGG-avidin the theoretical spacing of the biotinylated compounds has to be estimated and consequently adjusted to the required values.

4.3 Gelation properties and compression testing

One of the most crucial features for the final hydrogel product NaGG-avidin is, of course, its ability to form self-supporting gels. It has been well explored that commercial gellan gum can readily form gels with calcium [7], other mono- and divalent cations [12], as well as charged bioamines [17]. Here it has to be investigated whether the carried out functionalization perturbs the ability to form self-supporting, 'true' gels, and the compression behavior of the formed gels is analyzed.

Fig.15 shows photographs of gel samples with varying solvents, pH and gelation agents. From the photographs, different gel structures and qualities can be recognized. For instance, gels formed with SPM and SPD as gelation agent exhibit a rounded gel shape and are rather soft, whereas gels formed with calcium exhibit accurate edges and very good gel shape. All of the shown gel samples are self-supporting, clear and transparent. As a consequence of rapid gelation, gels formed with SPM may exhibit a white substance in the center.

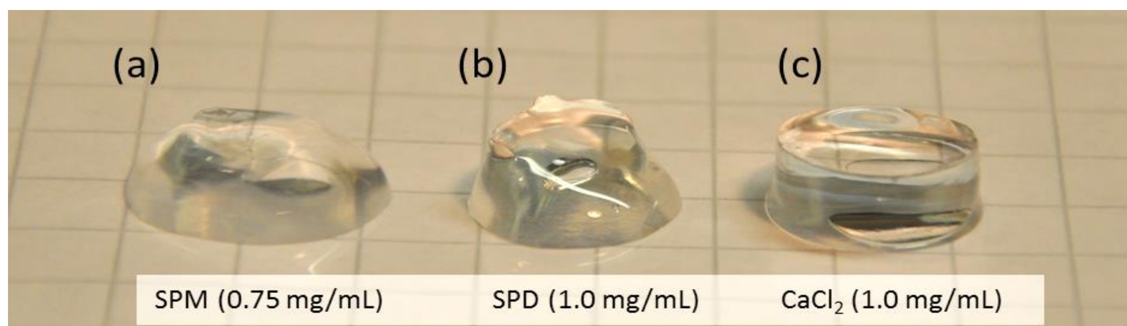

Figure 15. *Gel samples from NaGG-avidin in HEPES (50 mM, pH 6.5) with sucrose (10%).*

Deviating from the previous protocol for gel sample preparation, the NaGG-avidin samples were dissolved in a buffer solution, rather than water. It was observed that when the NaGG-avidin is dissolved in aqueous sucrose solution without buffer, the solution, as well as the gel samples, was not transparent but slightly turbid. The reason for this effect is unclear at the moment. Figure 16 shows a photograph of NaGG-avidin samples in HEPES pH 6.5, and demonstrates the transparency of the gel samples, regardless of gelation agent, sample quality and mechanical properties.

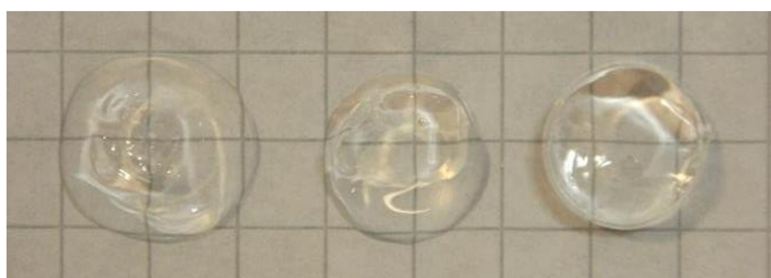

Figure 16. *Photograph illustrating the transparency of NaGG-avidin gel samples in HEPES/sucrose pH 6.5 with different gelation agents.*

The gelation time for NaGG-avidin gels is in the range of minutes. It is however difficult to determine an exact point of gel formation with the tilt-test. The quickest gelation observed was approximately one to two minutes for calcium cross-linked gels, whereas cross-linking with bioamines can take up to 20 minutes.

A range of different solvents and gelation agents were used during this project. Next to water, HEPES buffer with a concentration of 50 mM and varying pH was tested as solvent for gel formation. The pH of the HEPES solution ranged from 5.6 to 6.5 and 7.5. It was investigated whether an addition of 10% sucrose (1g/10mL) affects the gelation. An aqueous sucrose solution will be required in order to create an isotonic medium for the cell culture. Thus it needs to be verified that the presence of sucrose does not negatively affect the gel-formation. It was observed that dissolving NaGG-avidin in water and sucrose, without any buffer, results in non-transparent solution. The bioamines, SPM and SPD, are the desired gelation agents (refer to chapter 2.1.2), but nevertheless the gelation with calcium was also examined. Calcium chloride dihydrate was used in a concentration

of 1 mg/mL (25 mM), while the SPM concentration ranged from 0.35 to 1.40 mg/mL and the SPD concentration between 0.75 and 1.0 mg/mL.

Compression testing was carried out in order to quantify the gelation and mechanical properties of the gel samples. Here, the results are not shown as averages with variation, because not enough parallel samples with consistent sample quality could be prepared. This was due either because of little sample material, or inhomogeneous samples within one test series. For good practice and reliable results, it is desirable to measure five parallel gel samples and acquire their average. This was unfortunately not always possible, due to the large variation in sample quality and shape for samples of the same composition. One sample may have good shape and quality, whereas the next may be entirely fluid or have too poor shape to be tested. Repeatability, sample preparation and the mixing of gellan gum and gelation agent remain to be an issue. All shown curves (Fig. 16, 17 and 18) are representative examples, and not averaged data, due to the small amount of consistent parallel samples. The compression moduli are calculated based on the area the force is applied upon, meaning the cross-section of the cylinder-shaped samples. With poor sample shape (refer to Fig. 15) the samples deviate from ideal cylinder geometry and subsequently the calculated stress has to be understood with caution.

Gel samples were prepared from the sodium-purified gellan gum to verify that merely the purification does not inhibit or prevent the gelation with the previously studied gelation agents. Fig. 17 demonstrates NaGG samples with the three different types of gelation agents SPM, SPD and calcium. The calcium cross-linked gels are noticeably stronger compared to the bioamine versions.

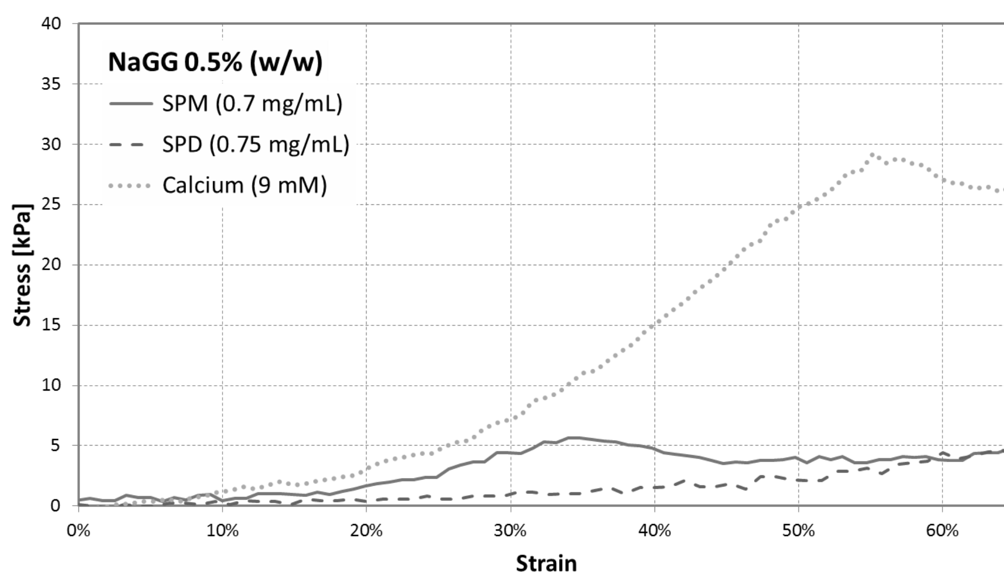

Figure 17. *Single result stress-strain curves of NaGG gel samples with SPM, SPD and calcium.*

Fig. 18 compares the compression behavior of gellan gum (GG) and sodium-purified gellan gum (NaGG). The GG and NaGG gels created with SPM are rather congruent and the depicted single results exhibit a compression modulus of 18.1 kPa and 20.2 kPa respectively. The gels formed with SPD show a greater variation and the compression modulus for NaGG is exceptionally high with 52.0 kPa compared to GG with 30.2 kPa. Strong gels like these were not obtained very commonly.

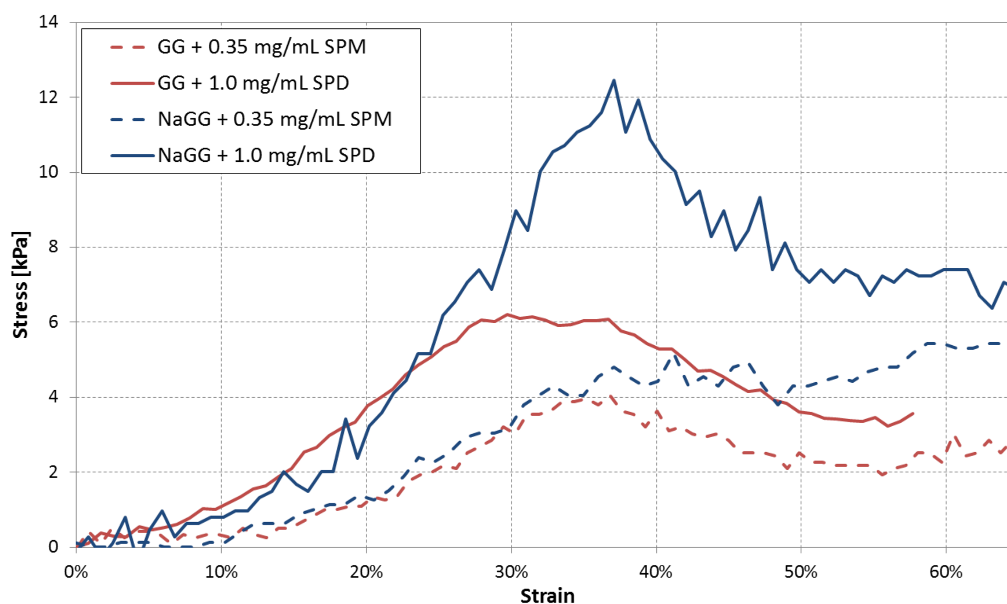

Figure 18. *Stress-strain curves of the following gel sample: GG (SPM 0.35 mg/mL) 18.1 kPa, GG (SPD 1.0 mg/mL) 30.2 kPa, NaGG (SPM 0.35 mg/mL) 20.2 kPa, NaGG-avidin (SPD 1.0 mg/mL) 52.0 kPa.*

The functionalized gellan gum samples (NaGG-avd) proved to form good and strong gels with calcium as cross-linker. The NaGG-avd sample with 0.35 mg/mL $CaCl_2$ shown in Fig 19 has a compression modulus of 116.4 kPa. However, using bioamines as gelation agent results in very soft gel samples, which commonly have poor sample shape, as can also be seen from the photograph in Fig. 15. The shown gel sample with spermine (SPM) has a modulus of 21.3 kPa, while the spermidine sample has a modulus of 7.1 kPa. Furthermore, an essential difference in compression behavior can be observed. Whereas GG and NaGG samples usually exhibit a distinct yield point, the shown NaGG-avd SPD sample has no breaking point within the measured 65% of compression, but rather shows a strain hardening effect. This behavior was observed frequently for NaGG-avd samples, for both SPD and SPM cross-linked versions.

Indeed, these two different compression behaviors are conspicuous and the samples were frequently observed to behave according to either category. GG and NaGG gel samples commonly exhibit a yield point around 20% to 50% and gel fracture occurs. However, the functionalized NaGG-avidin samples predominantly show this behavior of strain hardening, but exhibit no fracture point in the tested strain region. This type of compression behavior is also observed for biological, soft tissues like brain [73].

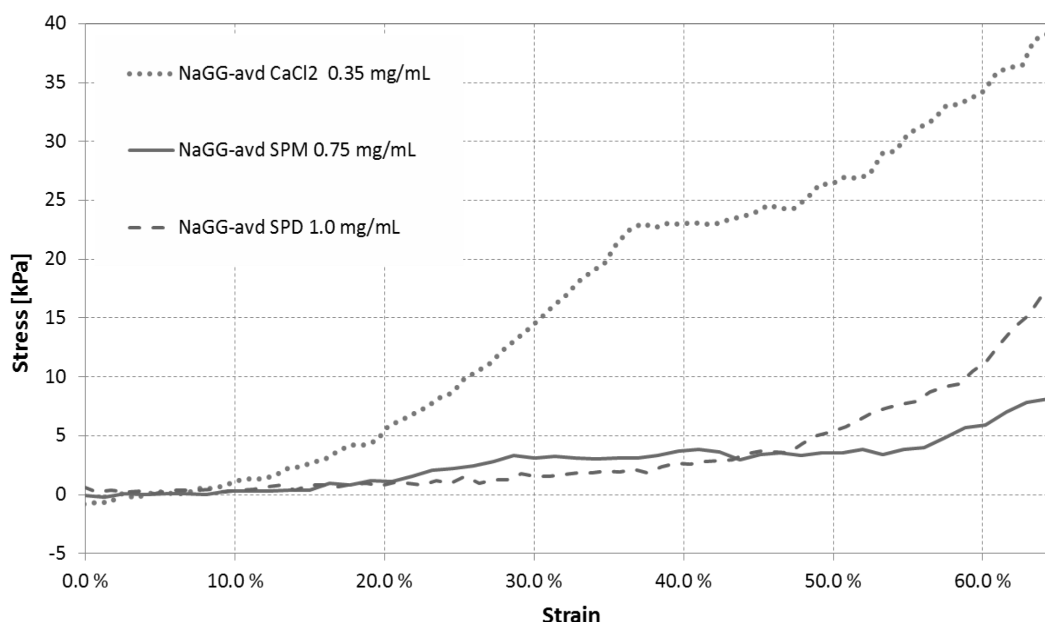

Figure 19. *NaGG-avd samples cross-linked with $CaCl_2$ (0.35 mg/mL, 116.4 kPa), SPM (0.75 mg/mL in HEPES pH 5.5, 21.3 kPa) and SPD (1.0 mg/mL in HEPES pH 5.5, 7.1 kPa).*

The effect of solvents, pH and sucrose addition was studied in more detail for NaGG-avidin gel samples. Once again a large variety in sample quality within parallel samples was observed, which results in a significant error for the calculated moduli. Additionally, the sample size was very low with two samples per type, due to the small amount of raw material available. Table 6 summarizes the average compression moduli of gel with different solvents, gelation agent and pH of the solution.

Table 6. *Compression moduli of NaGG-avidin gel samples in HEPES buffer with various pH, with or without sucrose, two different gelation agents; (a) average of two results (b) result of one single sample.*

NaGG-avidin		Gelation agent		Compression modulus [kPa]		
concentration [w/v]	**Solvent**	**type**	**concentration [mg/mL]**	**pH 5.5**	**pH 6.5**	**pH 7.5**
0.5 %	HEPES	SPM	0.75	26.8 ± 7.8 [a]	14.0 ± 6.0 [a]	3.8 [b]
0.5 %	HEPES	SPD	1.00	14.1 ± 0.9 [a]	5.1 ± 0.5 [a]	3.0 ± 0.9 [a]
0.5 %	HEPES/ sucrose	SPM	0.75	16.1 ± 0.3 [a]	17.8 [b]	2.9 ± 1.2 [a]
0.5 %	HEPES/ sucrose	SPD	1.00	10.4 ± 10.5 [a]	9.8 ± 5.1 [a]	4.6 ± 0.8 [a]

A few observations can be made from the compression testing of the samples detailed in Table 6. Due to the small sample size (n=2) these observations should be considered with caution. The compression moduli suggest that SPM creates stronger gels than SPD,

nevertheless the gel samples were observed to exhibit a greater homogeneity and shape when cross-linked with SPD. Additionally, SPD seems to act slower during gelation, likely due to its smaller net charge (refer to Fig. 3 in 2.1.1), and thus clear gels are created. Often with SPM a white substance is formed in the center of the gel, impairing the transparency of the gel. This is avoided with a lower SPM concentration, which however results in very poor sample shape and strength.

Besides gelation agent, the presence of sucrose appears to affect the response to compression. Gel samples in sucrose seem to exhibit the previously described elastic behavior more frequently and show no distinct yield point, compared to samples without sucrose.

A third effect that becomes noticeable is the increase in pH resulting in consistently lower compression moduli. Previously the unfunctionalized gellan gum samples were prepared in an aqueous sucrose solution which has a pH of around 5.5. Since the functionalized NaGG-avidin samples, however, do not dissolve into a transparent solution in pure water/sucrose, a buffer solution was used. The buffer is also expected to better accommodate the nChiAvd. Thus a pH range between the original 5.5 and a more biological pH of 7.5 was investigated. It is found that a more acidic medium is apt to result in tougher gels. It can be assumed that this is due to the bioamines, which rely on being fully protonated to aid the aggregation of gellan gum clusters and thus being more effective in an acidic environment. A low, acidic pH is, in turn, expected to be deterrent for any cell culture experiment. Since the pH of a cell culture is controlled by the culture medium it can only be speculated what magnitude of impact the initial pH of the matrix has on the cell line. Similarly, the effect of pH and ion concentration in the culture medium can be speculated to have an effect on the properties, especially the mechanical properties, of the hydrogel.

In order to achieve stronger gels it would be beneficial to heat the components to higher temperatures like 60°C before mixing them, in order to induce the helix formation of the polysaccharide chain [7]. This strategy has to be neglected for 3D cell culture applications, since the gel is intended to be seeded with cells, which cannot sustain these elevated temperatures. When considering 2D cell culture, the hydrogel can be casted at elevated temperature and cooled down, before the cells are seeded on the substrate's surface.

In summary it can be stated that the gelation of NaGG-avidin is possible and that the functionalization does not fundamentally alter the ability of gellan gum to form self-supporting hydrogels. Further investigation is required to determine the optimal solvent, buffer and pH to carry out gelation, as well as an in-depth study of the effect of bioamines as gelation agents. Perhaps it will be necessary to aid the cross-linking with a small amount of calcium, i.e. to use a mixture of spermine or spermidine and calcium chloride to achieve a good gel structure and desired mechanical properties.

5. CONCLUSIONS

The presented work shows that the polysaccharide gellan gum can be functionalized using carbodiimide coupling with EDC and NHS. The protein avidin is coupled covalently to the gel network and retains its ability to bind biotin. Moreover, the final product, NaGG-avidin, is able to form true gels with different gelation agents such as spermine, spermidine and calcium. The degree of functionalization was selected to be 2% of the amount of carboxyl groups of GG, which results in a concentration of 1.6 µg nChiAvd per 1 mg of NaGG. The gel samples showed a wide range of compression behavior and moduli, depending on gelation agent and concentration. The compression modulus has been observed to be as low as 2.9 kPa (0.75 mg/mL SPM) or reach values up to 116.4 kPa (0.35 mg/mL $CaCl_2$).

Functionalizing gellan gum will broaden its versatility for tissue engineering applications. This approach offers an off-the-shelf hydrogel construct that can readily be tailored to specific requirements. To add a desired functionality to the hydrogel, biotinylated compound, or a mixture of biotinylated compounds, are combined with the solution and immobilized through the avidin-biotin binding. Within the past 15 years an abundance of functionalized hydrogels has been presented in the literature. They, however, limit themselves to coupling one or two, specific cell cues to the hydrogel network, most prominently RGD peptide. In contrast, this approach provides a highly modular tool for cell research and tissue engineering.

Besides the biochemical environment guiding cell fate, there are other factors that need to be addressed for the successful design of matrix materials in 3D cell modeling and other tissue engineering applications. An essential parameter for cell culture is the mechanical behavior of the substrate, or the 3D hydrogel respectively. It has been shown that stiffness has a tremendous effect on cell morphology and differentiation. Furthermore, for studying cell behavior and modelling of tissues it is of great benefit if the artificial support structure, i.e. the hydrogel, is transparent and has good optical properties. This allows for conventional microscopic techniques to study cells after and during the cultivation process. Finally, the ability to form true, self-supporting gels is a key factor for the application of hydrogels in tissue engineering. Before any biomaterial can be considered for applications such as regenerative medicine its gel formation has to be verified and its mechanical properties need to be quantified. All of the mentioned issues have been discussed within this work. Further research on these hydrogels must include thorough assessment of compression results and the statistical handling of the data. The repeatability and reproducibility has to be confirmed through analytical means.

Despite the successful functionalization of GG and the proven capability to form gels, the implemented protocol leaves room for improvement. Most importantly it needs to be

determined how the NaGG-avidin samples can be gelated in a reliable fashion. The large variety in sample quality, also within samples of the same composition, indicates that practical issues, primarily the mixing of the different components, have a great effect on the final gel sample. Similarly, the type and concentration of gelation agent need to be adjusted. In case that a satisfying gel cannot be achieved with the current procedure, different functionalization strategies could be considered. An intriguing approach to assure the cross-linking of polysaccharides such as gellan gum is to avoid using the carboxyl groups for functionalization, since they are needed also for cross-linking. The end-group modification proposed by Bondalapati et al. poses an elegant method for the functionalization of polysaccharides [13]. The chemical conditions are, however, by far more severe and the used chemicals may be undesired if remaining, even in trace amounts, for biological applications. Succinctly, the advantages and disadvantages of different chemical strategies have to be carefully weighed.

Another interesting point that has not conclusively been determined is the ideal or desired degree of functionalization, which directly corresponds to the final avidin concentration in the hydrogel. Cells require a certain distance between adhesive cues and thus controlling the concentration of cues and available avidin-binding sites in the hydrogel is imperative. The next step for investigating the functionalized gellan gum will be to immobilize biotinylated RGD and observe the gelation. Moreover, it is planned to study the effect of the RGD-immobilized NaGG-avidin onto different types of cells.

REFERENCES

[1] R. Langer and D. A. Tirrell, "Designing materials for biology and medicine," *Nature,* vol. 428, pp. 487-492, 2004.

[2] F. Brandl, F. Sommer and A. Goepferich, "Rational design of hydrogels for tissue engineering:," *Biomaterials,* vol. 28, pp. 134-146, 2007.

[3] X. Li, E. Katsanevakis, X. Liu, N. Zhang and X. Wen, "Engineering nerual stem cell fates with hydrogel design for central nervous system regeneration," *Progress in Polymer Science,* vol. 37, pp. 1105-1129, 2012.

[4] C. K. Choi, M. T. Breckenridge and C. S. Chen, "Engineered materials and the cellular microenvironment: a strenghthening interface between cell biology and bioengineering," *Trends in Cell Biology,* vol. 20, no. 12, pp. 705-714, 2010.

[5] S. Breslin and L. O'Driscoll, "Three-dimensional cell culture: The missing link in drug discovery," *Drug Discovery Today,* vol. 18, no. 5/6, pp. 240-249, 2013.

[6] B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini and N. A. Peppas, "Hydrogels in Regenerative Medicine," *Advanced Materials,* vol. 21, pp. 3307-3329, 2009.

[7] E. R. Morris, K. Nishinari and M. Rinaudo, "Gelation of gellan - A review," *Food Hydrocolloids,* no. 373-411, p. 28, 2012.

[8] J. A. Rowley, G. Madlambayan and D. J. Mooney, "Alginate hydrogels as synthetic extracellular matrix materials," *Biomaterials,* vol. 20, pp. 45-53, 1999.

[9] D. F. Coutinho, S. V. Sant, H. Shin, J. T. Oliveira, M. E. Gomes, N. M. Neves, A. Khademhosseinic and R. L. Reisa, "Modified Gellan Gum hydrogels with tunable physical and mechanical properties," *Biomaterials,* vol. 31, no. 29, p. 7494–7502, 2010.

[10] C. J. Ferris, L. R. Stevens, K. J. Gilmore, E. Mume, I. Greguric, D. M. Kirchmajer, G. G. Wallace and M. i. h. Panhuis, "Peptide modification of purified gellan gum," *Journal of Materials Chemistry B,* vol. 3, p. 1106–1115, 2015.

[11] A. M. Fialho, L. M. Moreira, A. T. Granja, A. O. Popescu, K. Hoffmann and I. Sá-Correia, "Occurrence, production, and applications of gellan: current state and perspective," *Appl Microbiol Biotechnol,* vol. 79, p. 889–900, 2008.

[12] A. M. Smith, R. M. Shelton, Y. Perrie and J. J. Harris, "An Initial Evaluation of Gellan Gum as a Material for Tissue Engineering Applications," *Journal of Biomaterials Applications,* vol. 22, pp. 241-254, 2007.

[13] S. Bondalapati, E. Ruvinov, O. Kryukov, S. Cohen and A. Brik, "Rapid End-Group Modifi cation of Polysaccharides for Biomaterial Applications in Regenerative Medicine," *Macromolecular Rapid Communications,* vol. 35, p. 1754−1762, 2014.

[14] V. D. Prajapati, G. K. Jani, B. S. Zala and T. A. Khutliwala, "An insight into the emerging exopolysaccharide gellan gum as a novel polymer," *Carbohydrate Polymers,* vol. 93, p. 670–678, 2013.

[15] F. Kedzierewicz, C. Lombry, R. Rios, M. Hoffman and P. Maincent, "Effect of the formulation on the in-vitro release of propranolol from gellan beads," *International Journal of Pharmaceutics,* vol. 178, no. 1, pp. 129-136, 1999.

[16] T. Osmałek, A. Froelich and S. Tasarek, "Application of gellan gum in pharmacy and medicine," *International Journal of Pharmaceutics,* vol. 466, no. 1–2, p. 328–340, 2014.

[17] R. López-Cebral, P. Paolicelli, V. Romero-Caamaño, B. N. Seijo, M. A. Casadei and A. Sanchez, "Spermidine-Cross-linked Hydrogels as Novel Potential Platforms for Pharmaceutical Applications," *Journal of Pharmaceutical Sciences,* vol. 102, no. 8, pp. 2632-2643, 2013.

[18] A. Rozier, C. Mazuel, J. Grove and B. Plazonnet, "Gelrite®: A novel, ion-activated, in-situ gelling polymer for ophthalmic vehicles. Effect on bioavailability of timolol," *International Journal of Pharmaceutics,* vol. 57, no. 2, p. 163–168, 1989.

[19] L. W. Doner, "Rapid purification of commercial gellan gum to highly soluble and gellable monovalent cation salts," *Carbohydrate Polymers ,* vol. 32, pp. 245-247 , 1997.

[20] D. M. Kirchmajer, B. Steinhoff, H. Warren, R. Clark and M. i. h. Panhuis, "Enhanced gelation properties of purified gellan gum," *Carbohydrate Research,* vol. 38, pp. 125-129, 2014.

[21] J. Jänne, L. Alhonen and P. Leinonen, "Polyamines: from molecular biology to clinical applications," *Annals of Medicine,* vol. 23, no. 3, pp. 241-59, 1991.

[22] N. Cao, X. B. Chen and D. J. Schreyer, "Influence of Calcium Ions on Cell Survival and Proliferation in the Context of an Alginate Hydrogel," *ISRN Chemical Engineering,* vol. Volume 2012, no. Article ID 516461, 2012.

[23] ASTM Standard F2900−11, "Standard Guide for Characterization of Hydrogels used in Regenerative Medicine," ASTM Inernational, 2013.

[24] G. O. Phillips and P. A. William, Handbook of Hydrocolloids, Woodhead Publishing in Food Science, Technology and Nutrition, 2009.

[25] L. Salonen, *Rheological And Mechanical Properties Of Hydrogels,* Tampere: Tampere University of Technology, 2014.

[26] A. L. Berrier and K. M. Yamada, "Cell–Matrix Adhesion," *Journal of Cellular Physiology,* vol. 213, p. 565–573, 2007.

[27] J. L. Drury and D. J. Mooney, "Hydrogels for tissue engineering: scaffold design variables and applications," *Biomaterials,* vol. 24, p. 4337–4351, 2003.

[28] J. Connelly, T. Petrie, A. García and M. Levenston, "Fibronectin- And Collagen-Mimetic Ligands Regulate Bone Marrow Stromal Cell Chondrogenesis In Three-Dimensional Hydrogels," *European Cells and Materials,* vol. 22, pp. 168-177, 2011.

[29] N. J. Walters and E. Gentleman, "Evolving insights in cell–matrix interactions: Elucidating how non-soluble properties of the extracellular niche direct stem cell fate," *Acta Biomaterialia,* vol. 411, p. 3–16, 2015.

[30] X. Li, E. Katsanevakis, X. Liu, N. Zhang and X. Wen, "Engineering neural stem cell fates with hydrogel design for CNS regeneration," *Progress in Polymer Science,* vol. 37, p. 1105–1129, 2012.

[31] L. A. Reis, L. L. Chiu, Y. Liang, K. Hyunh, A. Momen and M. Radisic, "A peptide-modified chitosan–collagen hydrogel for cardiac cell culture and delivery," *Acta Biomaterialia,* vol. 8, p. 1022–1036, 2012.

[32] M. Tallawi, E. Rosellini, N. Barbani, M. G. Cascone, R. Rai, G. Saint-Pierre and A. R. Boccaccini, "Strategies for the chemical and biological functionalization of

scaffolds for cardiac tissue engineering," *Journal of the Royal Society Interface,* vol. 12, no. 108, 2015.

[33] S. Minatoguchi, G. Takemura, X.-H. Chen, N. Wang, Y. Uno, M. Koda, M. Arai, Y. Misao, C. Lu, K. Suzuki, K. Goto, A. Komada, P. Tomoyuki Takahashi, K. Kosai, T. Fujiwara and H. Fujiwara, "Acceleration of the Healing Process and Myocardial Regeneration May Be Important as a Mechanism of Improvement of Cardiac Function and Remodeling by Postinfarction Granulocyte Colony–Stimulating Factor Treatment," *Circulation,* vol. 109, pp. 2572-2580, 2004.

[34] L. Calvillo, R. Latini, J. Kajstura, A. LeriL, P. Anversa, P. Ghezzi, M. Salio, A. Cerami and M. Brines, "Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling," *Proceedings of the Natural Academy of Sciences of the United States of America,* vol. 100, no. 8, p. 4802–4806, 2003.

[35] A. M. Wobus, G. Kaomei, J. Shan, M.-C. Wellner, J. Rohwedel, J. Guanju, B. Fleischmann, H. A. Katus, J. Hescheler and W.-M. Franz, "Retinoic Acid Accelerates Embryonic Stem Cell-Derived Cardiac Differentiation and Enhances Development of Ventricular Cardiomyocytes," *Journal of Molecular and Cellular Cardiology,* vol. Volume 29, no. 6, p. 1525–1539, 1997.

[36] G. Nowak and R. G. Schnellmann, "L-ascorbic acid regulates growth and metabolism of renal cells: improvements in cell culture," *American Journal of Physiology - Cell Physiology,* vol. 271, no. 6, 1996.

[37] L. Pan, Y. Ren, F. Cui and Q. Xu, "Viability and Differentiation of Neural Precursors on Hyaluronic Acid Hydrogel Scaffold," *Journal of Neuroscience Research,* vol. 87, p. 3207–3220, 2009.

[38] W. K. Scholz, "Cell Adhesion and Growth on Coated or Modified Glass or Plastic Surfaces," ThermoScientific, 2010.

[39] X. He, J. Ma and E. Jabbari, "Effect of Grafting RGD and BMP-2 Protein-Derived Peptides to a Hydrogel Substrate on Osteogenic Differentiation of Marrow Stromal Cells," *Langmuir,* vol. 24, pp. 12508-12516, 2008.

[40] I. Jun, S. J. Kim, E. Choi, K. M. Park and T. Rhim, "Preparation of Biomimetic Hydrogels with Controlled Cell Adhesive Properties and Topographical Features for the Study of Muscle Cell Adhesion and Proliferation," *Macromolecular Bioscience,* vol. 12, p. 1502–1513, 2012.

[41] N. A. Silva, M. J. Cooke, R. Y. Tam, N. Sousa and A. J. Salgado, "The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate," *Biomaterials,* vol. 33, pp. 6345-6354, 2012.

[42] J. Zhu, C. Tang, K. Kottke-Marchant and R. E. Marchant, "Design and Synthesis of Biomimetic Hydrogel Scaffolds with Controlled Organization of Cyclic RGD Peptides," *Bioconjugate Chemistry,* vol. 20, p. 333–339, 2009.

[43] A. Mathur, A. Collinsworth, W. Reichert, W. Kraus and G. Truskey, "Endothelial, cardiac muscle and skeletal muscle exhibit different viscous and elastic properties as determined by atomic force microscopy.," *Journal of Biomechanics,* vol. 34, no. 12, pp. 1545-1553, 2001.

[44] L. Ylä-Outinen, T. Joki, M. Varjola, H. Skottman and S. Narkilahti, "Three-dimensional growth matrix for human embryonic stem cell-derived neuronal cells," *Journal of Tissue Engineering and Regenerative Medicine,* vol. 8, no. 3, pp. 186-94, 2014.

[45] S. S. Soofi, J. A. Last, S. J. Liliensiek, P. F. Nealey and C. J. Murphy, "The elastic modulus of Matrigel™ as determined by atomic force microscopy," *Journal of Structural Biology ,* vol. 167, no. 3, p. 216–219, 2009.

[46] Z. Drira and V. K. Yadavalli, "Nanomechanical measurements of polyethylene glycol hydrogels using atomic force microscopy," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 18, p. 20–28, 2013.

[47] R. Hobzova, M. Pradny, N. M. Zhunusbekova, J. Sirc, V. Guryca and J. Michalek, "Bioactive support for cell cultivation and potential grafting. Part 1 Surface modification of 2-hydroxyethyl methacrylate hydrogels for avidin immobilization," *e-Polymers,* vol. 043, 2011.

[48] G. T. Hermanson, "2. The Chemistry of Reactive Groups," in *Bioconjugate Techniques*, San Diego, CA, USA, Academic Press, 1996, p. 137 ff..

[49] Y. Miyagi, L. L. Chiu, M. Cimini, R. D. Weisel, M. Radisic and R.-K. Li, "Biodegradable collagen patch with covalently immobilized VEGF for myocardial repair," *Biomaterials,* vol. 32, pp. 1280-1290, 2011.

[50] Š. Kubinová, D. Horák, V. Vaněček, Z. Plichta, V. Proks and E. Syková, "The use of new surface-modified poly(2-hydroxyethyl methacrylate) hydrogels in tissue engineering: Treatment of the surface with fibronectin subunits versus Ac-

CGGASIKVAVS-OH, cysteine, and 2-mercaptoethanol modification," *Journal of Biomedical Materials Research A* , vol. 102, no. 7, pp. 2315-23, 2014.

[51] S. Woerly, "Restorative surgery of the central nervous system by means of tissue engineering using NeuroGel implants," *Neurosurgery Review,* vol. 23, p. 59–77, 2000.

[52] P. Vermette, T. Gengenbach, U. Divisekera, P. A. Kambouris, H. J. Griesser and L. Meagher, "Immobilization and surface characterization of NeutrAvidin biotin-binding protein on different hydrogel interlayers," *Journal of Colloid and Interface Science,* vol. 259, p. 13–26, 2003.

[53] J. F. Watts and J. Wolstenholme, An Introduction to Surface Analysis by XPS and AES, John Wiley & Sons, Ltd, 2003 .

[54] D. Jan Schröder, "Fluorescence Recovery after Photobleaching (FRAP) and its Offspring," Leica Microsystems, 23. 11. 2011. [Online]. Available: http://www.leica-microsystems.com/science-lab/fluorescence-recovery-after-photobleaching-frap-and-its-offspring/. [Accessed 15. 03. 2016].

[55] Thermo Fisher Scientific Inc., "Overview of ELISA," 2015. [Online]. Available: https://www.thermofisher.com/fi/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html. [Accessed 15. 03. 2016].

[56] N. Blau, M. Duran and K. M. Gibson, Laboratory Guide to the Methods in Biochemical Genetics, Berlin, Heidelberg: Springer-Verlag, 2008.

[57] BioRad, "Oriole Fluorescent Gel Stain," [Online]. Available: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10017295_Oriole_Instruction_Man.pdf. [Accessed 02. 02. 2016].

[58] J. R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer, 2006.

[59] J. T. Oliveira, L. Martins, R. Picciochi, P. B. Malafaya, R. A. Sousa, N. M. Neves, J. F. Mano and R. L. Reis, "Gellan gum: A new biomaterial for cartilage tissue engineering applications," *Journal of Biomedical Materials Research Part A,* vol. 93A, no. 3, p. 852–863, 2010.

[60] M. L. Oyen, "Mechanical characterisation of hydrogel materials," *International Materials Review,* vol. 59, no. 1, pp. 44-59, 2014.

[61] K. Nakamura, E. Shinoda and M. Tokita, "The influence of compressionvelocity on strength and structure for gellan gels," *Food Hydrocolloids,* vol. 15, no. 3, pp. 247-252, 2001.

[62] N. M. Green, "Avidin: 3. The Nature of the Biotin-Binding Site," *Biochemistry Journal,* vol. 89, no. 3, pp. 599-609, 1963.

[63] G. T. Hermanson, "13. Avidin - Biotin Systems," in *Bioconjugate Techniques*, San Diego CA, USA, Academic Press, 2013, pp. 570-592.

[64] M. Wilchek and E. A. Bayer, "Foreword and introduction to the book (strept)avidin–biotin system," *Biomolecular Engineering,* vol. 16, p. 1–4, 1999.

[65] Thermo Scientific, "Avidin-Biotin Technical Handbook," Thermo Fisher Scientific Inc., 2009.

[66] www.rcsb.org, "Avidin Ribbon Structure," [Online]. Available: http://www.rcsb.org/pdb/explore/explore.do?structureId=2AVI. [Accessed 03. 02. 2016].

[67] J. A. Määttä, Y. Eisenberg-Domovich, H. R. Nordlund, R. Hayouka, M. S. Kulomaa, O. Livnah and V. P. Hytönen, "Chimeric Avidin Shows Stability Against Harsh Chemical Conditions—Biochemical Analysis and 3D Structure," *Biotechnology and Bioengineering,* vol. 108, no. 3, pp. 481-490, 2011.

[68] S. Ray, R. T. Steven, F. M. Green, F. Höök, B. Taskinen, V. P. Hytönen and A. G. Shard, "Neutralized Chimeric Avidin Binding at a Reference Biosensor Surface," *Langmuir,* vol. 31, no. 6, p. 1921–1930, 2015.

[69] V. P. Hytönen, J. A. E. Määttä, T. K. M. Nyholm, O. Livnah, Y. Eisenberg-Domovich, D. Hyre, H. R. Nordlund, J. Hörhä, E. A. Niskanen, T. Paldanius, T. Kulomaa, E. J. Porkka, P. S. Stayton, O. H. Laitinen and M. S. Kulomaa, "Design and construction of highly stable protease resistant chimeric avidins," *The Journal of Biological Chemistry,* vol. 280, no. 11, p. 10228 –10233, 2005.

[70] Perkin Elmer, Analytical Methods for Atomic Absorption Spectroscopy, 2000.

[71] H. J. Gruber, M. Marek, H. Schindler and K. Kaiser, "Biotin−Fluorophore Conjugates with Poly(ethylene glycol) Spacers Retain Intense Fluorescence after Binding to Avidin and Streptavidin," *Bioconjugate Chemistry,* vol. 8, no. 4, p. 552–559, 1997.

[72] O. Bernstein-Levi, G. Ochbaum and R. Bittona, "The effect of covalently linked RGD peptide on the conformation of polysaccharides in aqueous solutions," *Colloids and Surfaces B: Biointerfaces,* vol. 137, p. 214–220, 2016.

[73] F. Pervin and W. W. Chen, "Dynamic mechanical response of bovine gray matter and white matter brain tissues under compression," *Journal of Biomechanics,* vol. 42, no. 6, p. 731–735, 2009.

[74] T. Führmann, R. Tam, B. Ballarin, B. Coles, I. E. Donaghue, D. v. d. Kooy, A. Nagy, C. Tator, C. Morshead and M. Shoichet, "Injectable hydrogel promotes early survival of induced pluripotent stem cell-derived oligodendrocytes and attenuates longterm teratoma in spinal cord injury model," *Biomaterials* , vol. 83, pp. 23-36, 2016.

[75] R. Y. Tam, M. J. Cooke and M. S. Shoichet, "A covalently modified hydrogel blend of hyaluronan–methyl cellulose with peptides and growth factors influences neural stem/progenitor cell fate," *Journal of Materials Chemistry,* vol. 22, pp. 19402-19411, 2012.

[76] A. Šturcová, G. R. Davies and S. J. Eichhorn, "Elastic Modulus and Stress-Transfer Properties of Tunicate Cellulose Whiskers," *Biomacromolecules,* vol. 6, no. 2, p. 1055–1061, 2005.

APPENDIX A: TABLE OF FUNCTIONALIZED HYDROGELS IN THE LITERATURE

Hydrogel	Introduced modification	Functionalization reaction	Investigated cell type	Source
Alginate (generalization to other polysaccharides)	G_4**RGD**Y	End-group modification of polysaccharides with aniline-catalyzed oxime formation	Fibroblasts	[13]
Gellan gum (NaGG)	G_4**RGD**SY	Carbodiimide coupling of RGD-peptide with spacer to carboxyl group of GG	Murine skeletal muscle C2C12 (anchorage dependent) and rat pheochromocytoma PC12	[10]
MC and HA (methylcellulose and hyaluronan)	Ac-G**RGD**S-PASSK-G4-SR-L6-R2KK(Ma-leimide)G, platelet-derived growth factor (**PDGF-A**) cytokine	Functionalization of MC with streptavidin, using thiol-maleimide coupling: MC is activated and thiol group is added, then coupling with (a) maleimide-RGD and (b) maleimide-streptavidin. Streptavidin is then used for further addition of biotinylated growth factor	oligodendrocyte progenitor cells (OPCs) derived from human induced pluripotent stem cells (hiPSC)	[74] and [75]
PLEOF (poly(lactide-co-ethylene oxide fumarate))	Az-mPEG-**BMP** and Ac-G**RGD**	Click-chemistry; BMP peptide = corresponds to residues 73-92 of the knuckle epitope of rhBMP-2	Bone marrow stromal cells (osteogenic differentiation)	[39]
poly(HEMA) (poly(2-hydroxyethyl methacrylate)) copolymerized with methacrylate	**Avidin** (covalent and physical attachment)	Carbodiimide coupling; only surface modification of hydrogel	Keratinocytes	[47]
TTA (tetronic tyramine)	Synthetic peptide G**RGD**G_5Y	Enzyme mediated polymerization, micropatterning with PDMS (polydimethylsiloxane) molds	Myoblasts (C2C12), human aortic smooth muscle cells (hAoSM)	[40]

Hydrogel	Introduced modification	Functionalization reaction	Investigated cell type	Source
poly(HEMA-AEMA) (copolymer of 2-hydroxyethyl methacrylate and 2-aminoethyl methacrylate)	Ac-CGGAS**IKVAV**S-OH (laminin derived peptide) and Fibronectin Subunits (**Fn**),	Maleimide thiol coupling; Functionalization also with β-mercaptoethanol and cysteine, but just as comparison to peptides, but they showed no effect	Mesenchymal stem cells (MSCs) (study done to improve spinal cord injury)	[50]
Collagen (biodegradable patch)	**VEGF** (vascular endothelial growth factor)	Carbodiimide coupling	*in vitro*: Endothelial cells Bone marrow cells (BMCs) *in vivo*: right ventricular free wall in rats	[49]
HA (hyaluronic acid)	poly-L-**lysine** and **anti-NgR** *"anti-NgR (NgR-Ab) is an inhibitor of the Nogo complex of myelin-associated proteins, that prevents their interference with the axon outgrowth"*	Condensation reaction between aldehyde group of the antibody and hydrazide group of the HA hydrogel	Neural Precursor Cells (NPC) (from E13.5 forebrain cortical neuroepithelium display attachment)	[37]
Blend of Chitosan and Collagen	QH**REDG**S (cell-protective peptide, derived from protein growth factor Angiopoietin-1)	Carbodiimide coupling; First step: peptide modification of chitosan, second step: hydrogel formation with collagen	*in vitro*: Cardiomyocytes from neonatal Sprague–Dawley rat hearts *in vivo*: studies on rats (or mice?)	[31]
Alginate	G**RGD**Y	Carbodiimide coupling; Functionalization before and after gelation	C2C12 skeletal myoblasts	[8]
Gellan gum (furan modified)	G**RGD**S (derived from fibronectin), but also cultured with **OEG** (olfactory ensheathing glia)	Click-chemistry: Furan-modification on GG, maleimide-coupled peptide, coupling of both with Diels-Alder reaction	neural stem/ progenitor cell (NSPC)	[41]
PEGDA (poly(ethylene glycol) diacrylate)	cyclic **RGD** peptide with spacer (c[**RGD**fE(SSSKK-NH2))	NHS-modified polymer (end group) reacts with primary amine group of peptide (K) UV cross-linking of modified polymer	Human pulmonary artery endothelial cells	[42]

APPENDIX B: TABLE OF USED MATERIALS AND CHEMICALS

Chemical	Abbreviation	Source	Detail	Lot number
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	EDC	Sigma Aldrich		BCBL5597V
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid	HEPES	Sigma Aldrich	pH adjustment with NaOH to 5.5…7.5, biocertified H4034	SLBL6396V
β-mercaptoethanol		Sigma Aldrich	for molecular biology andelectrophoresis, 99%	BCBL6953V
Biotin-5-fluorescein	B5f	Sigma-Aldrich	10 µl aliquots in DMSO, product no. 53608	
Calcium carbonate	$CaCO_3$	Alfa Aesar		Y26A015
Calcium chloride dihydrate	$CaCl_2$ (x $2H_2O$)	Riedel-de Haën		1254A
D-Biotin		Fluka Chemie GmbH		
de-ionized water (millipore)	H_2O	-		
Dowex cation exchange resin		Sigma Aldrich	H+ form, 50–100 mesh, X8	MKBV5978V
GelzanTM, gellan gum	GG	Sigma Aldrich		SLBB0376V
Hydrochloric acid	HCl	-	1N	
Hydrogen peroxide	H_2O_2	Sigma Aldrich	50% solution	
Isopropyl alcohol	i-propanol	VWR chemicals	Product no. 20922.364	14D070506
N-Hydroxysuccinimide	NHS	Pierce	24500	HC102040
Neutralized chimeric avidin	CNCA, nChiAvd	BioMediTech	In dialysis buffer: 50 mM NaPO4, 100 mM NaCl, pH 7.5	

Chemical	Abbreviation	Source	Detail	Lot number
Potassium chloride	KCl	J.T. Baker, Avantor Performance Materials		0210220003
Sodium chloride	NaCl	J.T. Baker, Avantor Performance Materials		12223030006
Sodium hydroxide solution	NaOH	Merck KGaA, Emsure (R)	1M	
Spermidine trihydrochloride	SPD	Sigma Aldrich		BCBJ3890V
Spermine tetrahydrochloride	SPM	Sigma Aldrich		BCBK9212V
Sucrose		Sigma Aldrich		SLBF7618V
Sulfuric acid	H_2SO_4	Sigma Aldrich		SZBE2510V

APPENDIX C: EFFECTS OF SOLVENT ON GELLAN GUM AND AVIDIN

NaGG (wt%)	Solvent	Concentration	pH	Observation
1.0%	$NaPO_4$/NaCl	50 mM/ 100 mM	7.5	Does not dissolve, partial gelation, fibrous substance in solution
1.0%	MES/NaCl	50 mM/ 100 mM	6.5	gelation, incomplete dissolution
1.0%	MES	50 mM	6.5	dissolves easily, very viscous when cooled down, but no gelation
0.5%	$NaPO_4$/NaCl	25 mM/ 50 mM	7.6	dissolves somewhat, but seems to gelate
1.0%	HEPES/NaCl	50 mM/ 10 mM	7.5	dissolves after a while at ~50C, very viscous at RT, but no gelation
1.0%	HEPES/NaCl	50 mM/ 25 mM	7.5	highly viscous at RT, slightly gelated, but becomes more fluid when re-heated
1.0%	HEPES/NaCl	50 mM/ 20 mM	7.5	fluid at 45°C, but gelates quickly upon cooldown

Avidin	Solvent	Concentration	pH	Observation
nChiAvd	water		~5.5	precipitation
nChiAvd	MES	50 mM	6.5	about 60 % of protein precipitated during buffer change
nChiAvd	MES/NaCl	50 mM/ 100 mM	6.5	protein stayed mostly as tetramers
nChiAvd	$NaPO_4$/NaCl	50 mM/ 100 mM	6.0	protein stayed mostly as tetramers
nChiAvd	$NaPO_4$/NaCl	50 mM/ 100 mM	6.5	protein stayed mostly as tetramers
nChiAvd	$NaPO_4$/NaCl	50 nM/ 100 mM	7.0	protein precipitated
nChiAvd	$NaPO_4$/NaCl	50 nM/ 100 mM	7.5	protein stayed mostly as tetramers
nChiAvd	$NaPO_4$/NaCl	50 nM/ 100 mM	8.0	protein stayed mostly as tetramers
nChiAvd	HEPES	25 mM		DLS instrument error = cannot be measured, no result
nChiAvd	HEPES/NaCl	25 mM/ 25 mM	7.5	protein seems to be tetramer

APPENDIX D: CALCULATION OF CARBOXYL GROUPS IN GELLAN GUM

Molar mass of saccharide subunits in gellan gum

M(Rhamnopyranose)	164.16	g/mol
M(Glucapyranose)	180.16	g/mol
M(Glucoronic acid)	194.14	g/mol
Total M(repeat unit)	**718.62**	**g/mol**

Molar mass of nChiAvd (monomer 14.324 kDa)

M(Avidin)	5.73E+04	g/mol

Concentration of gellan gum

c (GG)	10	mg/mL
	0.01	g/mL

Amount of (COOH) → One carboxyl group in repeat unit and thus one in 718.62 g/mol.

$$n(\mathrm{COOH}) = \frac{c(\mathrm{GG})}{M(\text{repeat unit})}$$

n(COOH)	**1.39E-05**	**mol/mL**

Desired degree of functionalized carboxyl groups is 2%

(see discussion in 4.2)

Functionalization	2.00 %	of n(COOH)
c(avidin)	2.78E-07	mol/L

Volume of the GG used for functionalization (10 mg/mL)

in	10	ml of GG solution
V(GG)	0.01	L

$$n(\mathrm{avidin}) = \frac{c(\mathrm{avidin})}{V(\mathrm{GG})}$$

$$m(\mathrm{avidin}) = n(\mathrm{avidin}) \cdot M(\mathrm{avidin})$$

n(avidin)	2.78E-09	mol
m(avidin)	1.59E-04	g

m(avidin)	**0.16**	**mg of avidin per 10 ml of GG (10 mg/mL)**

APPENDIX E: PROTOCOL FOR PURIFICATION OF GELLAN GUM

Gelzan™ is purified to NaGG, in order to remove counterions (Ca^{2+}, Mg^{2+}) which can induce gelation. The only remaining counterions should be Na^{+}.

Dissolve 0.5 g GG (Gelzan™) in 100 ml DI water (gives: 5 mg/mL, 0.5% w/v) and heat the mixture to 60°C under constant stirring. Add 1.5 g Dowex cation exchange resin (H+ form, 50–100 mesh, prerinsed [1]). Stir the mixture for 30 min in a water bath at 60°C.

Stop the magnet stirrer (after the 30 min) and the resin settles. *In the meantime, another flask is prepared on a water bath at 60°C into which the filtrate will be transferred in order to prevent the solution from cooling down.* The supernatant is transferred with a plastic pipette onto a filter with rough filter paper (grade 4). The filter paper has to be changed a couple of times. To maximize the yield, the resin can be washed with DI water and the filtrates can be combined. The filtrate is kept at 60°C in water bath.

Add NaOH (1 N) dropwise to the filtrate until the pH reaches 7.5 and the solution is stirred for further 5 min. Be very careful when adding the NaOH, because the pH will change very suddenly. Test the pH with pH-test-strips[2].

Finally, pour small amounts of the solution into separate portions of 100 mL isopropanol in order to precipitate the NaGG. The large precipitate clusters are removed with the sieve from the isopropanol and dried on the vacuum filter. *(No need to pull the whole amount of isopropanol through the vacuum pump → vacuum filtration of volatile organic solvents is usually avoided).*

Collect all NaGG precipitate in a plastic tube and freeze overnight. Lyophilize the product over 2 days.

[1] Some articles call for the exchange resin to be pre-rinsed in HCl (1 M) and DI water. Presumably, this means the resin is transferred to a filter and flushed with a few milliliters of first HCl and afterwards water

[2] Using a pH electrode is also possible, but cleaning the electrode will require more work.

APPENDIX F: PROTOCOL FOR FUNCTIONALIZATION OF SODIUM-PURIFIED GELLAN GUM

Dissolve chopped NaGG (100 mg) in buffer (HEPES 50 mM, 10 ml, pH 6.5[3]). Heating in water bath at 60°C, stirring with a magnet stirrer. Once the NaGG is dissolved reduce water bath temperature to 40°C

Weigh in EDC (0.3 M, 50 μL → 28.8 mg in 0.5 mL) in the glove box while the NaGG is dissolving. Also prepare the NHS (0.15 M, 50 μL → 18.1 mg in 1.0 mL). Dissolve both EDC and NHS in buffer (HEPES 50 mM). Add 50 µl of EDC and consequently NHS to the dissolved NaGG. Stir for 15 min in water bath at 40°C.

Quench with β-mercaptoethanol (14µl, final concentration 20 mM). Stir for a few more minutes.

Add 45 µl nChiAvd (neutralized chimeric avidin, 3.540 mg/mL, 0.062 mM) and stir for 5 h.

Transfer solution with a pipette into a dialysis bag (MWCO 1214 kDa) and dialyze against DI water over five days. Monitor the pH of the dialysis water. *Dialysis eliminates unreacted components (EDC, NHS, etc), solvent (HEPES) and also purifies the product. If this step is carried out, also the original GG:cross-linker ratio can be maintained.*

Freeze the solution and lyophilize for two days.

[3] The pH of the solution and effect onto gel strength has been discussed in the thesis