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Development and *In vitro* Evaluation of an RGD-Functionalized Chitosan Derivative for Wound Healing

THÈSE

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par

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Sävedalen (Suède)

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Développement et évaluation *in vitro* d'un dérivé de chitosan fonctionnalisé avec des peptides RGD pour la cicatrisation

Biopharmacie, Ecole de pharmacie Genève-Lausanne, Université de Genève, Université de Lausanne, Suisse

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L'objectif du travail présenté dans cette thèse était de développer des nanoparticules fonctionnelles ayant la capacité d'induire l'adhésion et la migration de kératinocytes humains normaux. L'utilisation de systèmes particulaires pour favoriser l'adhésion et la migration cellulaire dans les processus de cicatrisation constitue une nouvelle approche de l'ingéniérie tissulaire.

Dans cette optique, un dérivé hydrosoluble du chitosan rendu fonctionnel par l'ajout de peptides RGD a été développé. Les nanoparticules furent développées par coacervation complexe entre le dérivé cationique du chitosan et le sulfate de chondroïtine anionique. La capacité du système particulaire à induire un changement cellulaire phénotypique a été évaluée *in vitro*.

Lors de l'évaluation de ce nouveau polymère, le succès de la synthèse a été montré par l'absence de cytotoxicité et par la préservation de son activité biologique médiée par les séquences RGD. Aussi bien les polymères que les nanoparticules ont induit l'adhésion et la mobilité de fibroblastes dermiques humains, confirmant le concept de nanoparticules bio-actives. Cependant, concernant l'étude des interactions entre les nanoparticules et les kératinocytes, aucune conclusion n'a pu être tirée et d'autres travaux sont nécessaires.

Pour résumer, un système particulaire bio-actif a été développé. Le choix des peptides RGD pour induire la migration des kératinocytes doit être réévalué, et l'utilisation de concentrations plus importantes, de mélange de peptides d'adhésion ou l'utilisation de peptides d'adhésion différents doit être envisagée pour la réalisation d'études ultérieures.

Mots clés: RGD ; chitosan ; nanoparticules ; adhésion cellulaire ; cicatrisation.

Development and *In* vitro Evaluation of an RGD-Functionalized Chitosan Derivative for Wound Healing

Biopharmaceuticals, School of Pharmacy, University of Geneva, University of Lausanne

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The aim of the work presented in this thesis, was to develop functionalized nanoparticles with the ability to induce adhesion and migration in normal human keratinocytes. Using particulate systems to promote and support cell adhesion and migration in epidermal restoration is a novel approach of tissue engineering.

In this view, a water-soluble chitosan derivative functionalized with RGD peptides was developed. Nanoparticles were formed through complex coacervation between the cationic chitosan derivative and the anionic chondroitin sulfate. The particulate system was evaluated *in vitro* for its ability to change phenotype in cells.

In the evaluation of the novel hybrid polymer, the successful synthesis was confirmed by the absence of cytotoxicity and a preserved bioactivity specific to the RGD-moieties. Both the polymer and the particles formed thereof induced cell adhesion and spreading in human dermal fibroblasts, proving the concept of bioactive nanoparticles. However, when investigating the interaction between the nanoparticles and keratinocytes, no clear conclusion could be drawn and further assays are required.

To summarize, a bioactive particulate system was developed. The choice of RGD peptides to induce migration in keratinocytes needs to be re-evaluated and higher concentrations, mixtures of adhesion peptides or other adhesion peptides might be considered for further investigations.

Keywords: RGD; chitosan; nanoparticles; cell adhesion; wound healing

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Foreword

The skin is our largest organ, an outer envelope defending us against mechanical stress, temperature changes and infectious agents. Upon wounding the protection is compromised and, in order to restore the barrier, an efficient repair system is pivotal.

Under normal conditions, wound healing is a spontaneous process induced upon wounding in which four overlapping phases might be distinguished: (i) hemostasis, (ii) inflammation, (iii) proliferation and (iv) remodeling (Singer and Clark, 1999). One of the cornerstones of the wound healing process is re-epithelialization. During the proliferation step, the keratinocytes at the wound edges and in the hair follicles proliferate and become migratory to successively crawl in over the wound bed to restore the epidermal barrier (Ortonne et al., 1981). This process is governed by insoluble structural features of the extracellular matrix and by soluble factors such as growth factors and cytokines (Li et al., 2004).

Wound healing is generally spontaneously induced upon wounding and terminated within a few days up to a few weeks (Stadelmann et al., 1998). However, if the repair process is impaired, complex, chronic wounds are formed and strategies for tissue regeneration may be required. With an aging population, and risk factors such as diabetes and obesity becoming more common, the rate of chronic wounds is increasing (Sen et al., 2009). Today there is no efficient treatment to induce healing in these wounds and new strategies are needed. In this thesis a novel approach to direct tissue restoration of the epidermis by using a particulate system functionalized with extracellular matrix (ECM) derived peptides is presented.

Adhesion peptides derived from the extracellular matrix (ECM) are small amino acid sequences eliciting a cell response similar to proteins, i.e. adhesion, spreading and migration. Although peptides are less specific, and require higher concentrations than the parent proteins, they are advantageous considering the ease of synthesis and their stability. Sequences of amino acids such as YIGSR (Graf et al., 1987), GFOGER (Knight et al., 2000) and RGD (Pierschbacher and Ruoslahti, 1984) from laminin, collagen and fibronectin, respectively, were shown to induce adhesion in

cells. RGD is the most investigated adhesion peptide, known to interact with a number of cell surface receptors belonging to the integrin family. Integrins are transmembrane cell surface receptors, mediating signals between the ECM and the cytoskeleton (Hynes, 2002). Upon binding to the receptor, members of the family of Rho GTPases are activated, inducing a re-arrangement of the cytoskeleton and subsequently the attachment and spreading of the cells (Margadant et al., 2010).

In order to induce attachment, adhesion peptides need to be anchored to a solid matrix. This can typically be achieved through grafting the peptide to a polymeric backbone. Chitosan is a biopolymer that has gained interest for biomedical and pharmaceutical applications due to its biocompatibility and the ease to modify its structure by adding functional groups (Alves and Mano, 2008; Baldrick, 2010). Particulate systems of chitosan have been used in mucosal drug delivery because of its mucoadhesive properties (Sinha et al., 2004). In addition, chitosan has recently been evaluated for scaffolds in tissue engineering (Miranda et al., 2011).

The general objective of this thesis was to develop nanoparticles decorated with RGD peptides, aimed at accelerating re-epithelialisation during wound healing in skin. This concept was based on two hypotheses, (i) the induction of change in phenotype of keratinocytes by RGD-peptides and (ii) the presentation of the peptides by mucoadhesive nanoparticles lining the wound bed supporting migrating cells. A schematic figure of the concept is shown in Fig. 1.

The realization of the present work has been divided into four chapters. The role of RGD-functionalized biomaterials in wound healing hitherto, with focus on its impact on the dermal fibroblasts and the epidermal keratinocytes, is reviewed in Chapter 1. The synthesis, characterization and *in vitro* evaluation of a chitosan derivative functionalized with RGD peptides are described in Chapter 2.

In Chapter 3, the preparation of nanoparticles through complexation between the RGD-functionalized chitosan derivative and chondroitin sulfate, the evaluation of their properties and their ability to support adhesion and induce cell spreading is assessed. Finally, the interaction between RGD-functionalized carboxymethyl trimethyl chitosan and keratinocytes is investigated in Chapter 4.

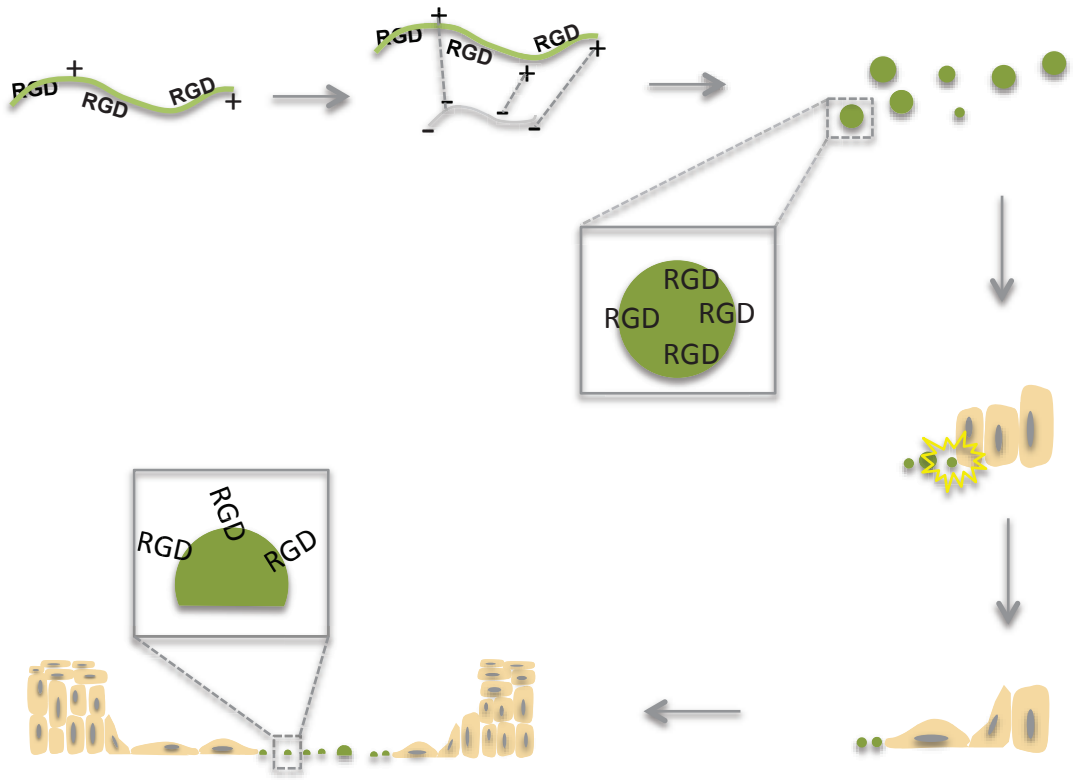


Figure 1. Schematic figure of the principle of nanoparticle-promoted wound healing. In a first step, cationic polymers functionalized with RGD peptides are synthesized. The cationic nature of the polymer allows nanoparticle formation through complex coacervation with a polyionic polymer. Bioactive nanoparticles are presented to stationary keratinocytes. Through an integrin-mediated interaction between RGD and the cells, the keratinocytes change their phenotype and become migratory. The keratinocytes crawl in over the wound bed and accelerate the healing process, ultimately restoring the epithelial barrier.

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Avant-propos

La peau représente l'organe le plus grand de l'organisme. Elle constitue une enveloppe externe qui nous protège des agressions mécaniques, des changements de température et des agents infectieux. Les blessures compromettent cette protection et un système de réparation efficace est nécessaire pour restaurer l'effet barrière de la peau.

En conditions normales, suites à une blessure, la cicatrisation se déroule selon quatre phases qui se chevauchent : hémostase (i), inflammation (ii), prolifération (iii) et (iv) remodelage (Singer et Clark, 1999). La re-épithélialisation constitue l'élément essentiel du processus de cicatrisation. Durant l'étape de prolifération, les kératinocytes présents sur les bords de la plaie et dans les follicules pileux acquièrent la capacité de migrer (Ortonne et al., 1981). Ce processus est gouverné par les composants insolubles de la matrice extra-cellulaire et des facteurs solubles tels que les facteurs de croissance et les cytokines (Li et al., 2004).

La cicatrisation s'active généralement de façon spontanée à la suite d'une blessure et dure de quelques jours à quelques semaines (Stadelmann et al., 1998). Cependant, quand le processus de réparation est défectueux, la plaie devient chronique et de stratégies de régénération tissulaire sont nécessaires. L'incidence des plaies chroniques augmente avec le vieillissement de la population et la généralisation des facteurs de risque tels que le diabète et le surpoids (Sen et al., 2009). A ce jour, aucun traitement efficace n'induit la cicatrisation et le développement de nouvelles stratégies thérapeutique s'avère donc nécessaire. Cette thèse présente une nouvelle approche pour guider la cicatrisation de l'épiderme en utilisant un système particulaire rendu fonctionnel par l'ajout de peptides dérivés de la matrice extra-cellulaire (MEC).

Tout comme les protéines, les peptides d'adhésion dérivés de la MEC sont de petites séquences d'acides aminés capables d'induire des réponses cellulaires telles que l'adhésion, l'étalement et la migration. Bien que les peptides soient moins

spécifiques et nécessitent des concentrations plus importantes que les protéines, ils se distinguent positivement par leur facilité de synthèse et leur stabilité. Il a été démontré que les séquences d'acides aminés telles que YIGSR (Graf et al., 1987), GFOGER (Knight et al., 2000), et RGD (Pierschbacher et Ruoslahti 1984) dérivées respectivement de la laminine, du collagène et de la fibronectine sont capables d'induire l'adhésion cellulaire. Connu pour interagir avec un certain nombre de récepteurs membranaires appartenant à la famille des intégrines, le RGD est un des peptides d'adhésion les plus étudiés. Les intégrines sont des récepteurs transmembranaires transmettant les signaux entre la MEC et le cytosquelette (Hynes 2002). Une fois le récepteur stimulé, les membres de la famille Rho des GTPases sont activés et induisent une réorganisation du cytosquelette, rendant la cellule capable d'attachement et de mobilité (Margadant et al., 2010). Les peptides d'adhésion doivent être solidement ancrés à une matrice pour induire l'attachement. Cette matrice peut être constituée par une molécule polymérique. Le chitosan est un polymère biologique d'intérêt médical et pharmaceutique croissant en raison de sa biocompatibilité et de la facilité de greffage de groupements fonctionnels (Alves et Mano 2008, Baldrick 2010). Du fait de ses propriétés mucoadhésives, des systèmes particulaires de chitosan ont été utilisés pour des médicaments administrés par application mucosale. De plus, particules de chitosan a été évalué comme support de régénération pour l'ingeniérie tissulaire (Miranda et al., 2011).

L'objectif général de cette thèse était de développer des nanoparticules liées à des peptides RGD, destinées à accélérer la ré-épithélialisation durant la cicatrisation. Ce concept est basé sur deux hypothèses (i) l'induction d'un changement phénotypique des kératinocytes par les peptides RGD, (ii) la présentation de peptides induisant la migration cellulaire par les nanoparticules muco-adhésives tapissant la plaie. Ce concept est schématisé dans la figure 1.

La réalisation de ce travail a été divisée en quatre parties. Le rôle des peptides RGD dans les processus de cicatrisation, notamment leur impact sur les fibroblastes et les kératinocytes, est décrit dans le premier chapitre. La synthèse, la caractérisation et l'évaluation *in vitro* du chitosan rendu fonctionnel par l'ajout de peptides RGD sont présentées dans le deuxième chapitre. Le troisième chapitre traite de la préparation des nanoparticules par complexation entre le chitosan et le sulfate de chondroïtine, ainsi que de l'évaluation de leurs propriétés et de leur capacité à induire l'adhésion

et la migration cellulaire. Finalement, les interactions entre le carboxymethyl-trimeyhyl chitosan fonctionnalisé avec des peptides RGD et les kératinocytes sont étudiées dans le quatrième chapitre.

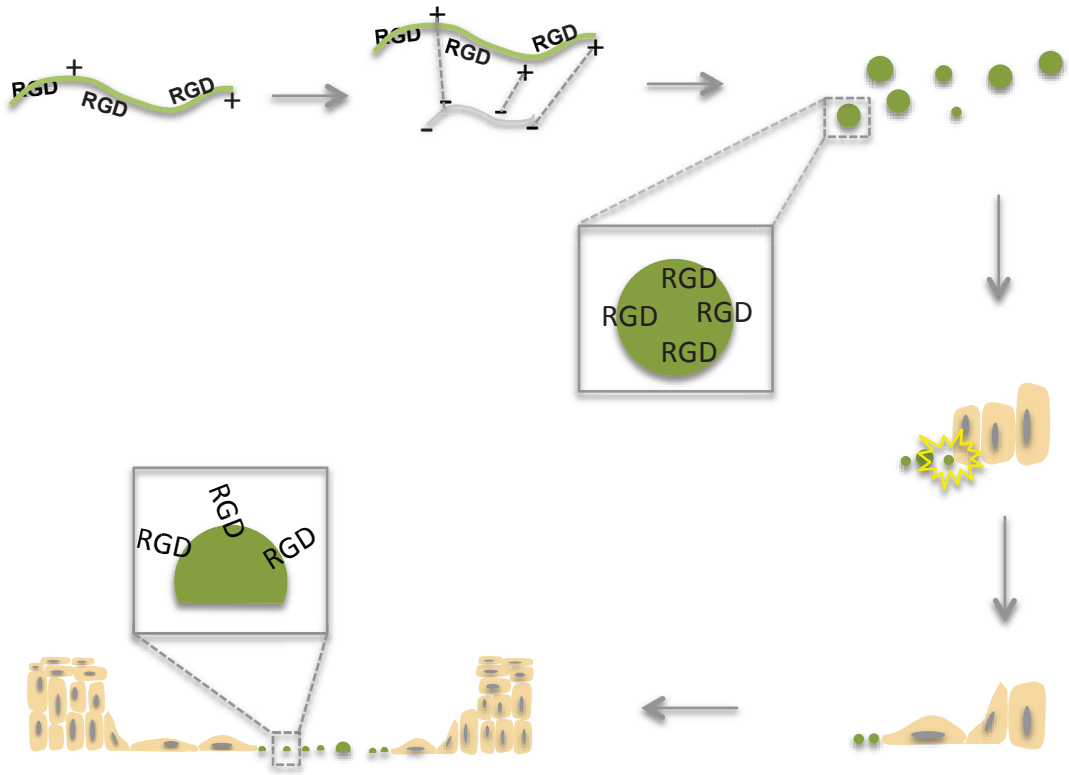


Figure 1. Représentation schématique du principe de la cicatrisation favorisée par des nanoparticules. Dans une première étape, les cations polymériques rendus fonctionnel par les peptides RGD sont synthétisés. La nature cationique du polymère permet la formation de nanoparticules par coacervation complexe avec un polymère polyionique. Les nanoparticules bioactives sont présentées à des kératinocytes immobiles. Les kératinocytes changent de phénotype et deviennent mobiles après interaction entre leurs récepteurs intégrines et le peptide RGD. Les kératinocytes migrent au cœur de la plaie et accélèrent la cicatrisation, restaurant finalement la barrière épithéliale.

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Chapter I:

RGD-Functionalized Biomaterials for Enhanced Interaction with Skin Cells in Wound Healing

To be submitted

Wound healing is a spontaneous process, temporarily and spatially closely controlled. In larger wounds or in complex wounds, external help might be needed to create a favorable wound environment and promote healing while protecting the underlying tissue. Polymeric biomaterials have been shown to be suitable for wound dressings as well as scaffolds for *in vitro* or *in vivo* colonization of skin cells. A favorable environment for adhesion, migration and subsequently colonization is pivotal for tissue repair and tissue regeneration. This is achieved through mimicking the extracellular matrix (ECM) by adding bioactive components derived from the ECM, such as proteins, peptides, glycosaminoglycans (GAGs) or growth factors. RGD is a small peptide sequence identified as the major cell binding sequence in the ECM component fibronectin. RGD has been investigated for its use to promote cell adhesion to biomaterials and successive migration. This review gives an overview of the use of RGD-functionalized biomaterials in applications aimed at enhancing wound healing of the dermis and epidermis. Current knowledge on the interaction between fibroblasts and keratinocytes with RGD *in vitro* and *in vivo* is reported, and new therapeutic strategies based on this knowledge highlighted.

Keywords: RGD peptide; biomaterial; wound healing; dermal fibroblast; keratinocytes

1. Introducution

The possibility of using short amino acid sequences in tissue engineering has been investigated since the early nineteen-eighties, when the cell binding sequences of fibronectin were identified and found to initiate a similar cell response as the whole protein. RGD-containing sequences have been extensively investigated *in vitro* and *in vivo* for various tissue engineering applications. Aiming at reconstructing the dermal and epidermal barrier, RGD has been tested to increase the cell-biomaterial interaction between skin cells and skin substitutes. Herein we summarize the reported work on RGD-modified biomaterials, *in vitro* and *in vivo*, with two of the major cell types re-building the skin after injury, fibroblasts and keratinocytes.

In general, wound healing is divided into four overlapping phases, starting immediately with the coagulation, closely followed by an inflammation phase, a phase of proliferation, and finally scar remodelling (Singer and Clark, 1999), as shown in Fig. 1. Diverse types of cells are involved in tissue repair, including various immune cells cleaning up the wound site, endothelial cells participating in angiogenesis, dermal fibroblasts rebuilding the extracellular matrix (ECM) and epidermal keratinocytes restoring the epithelium and the cutaneous barrier (Midwood et al., 2004). The main cell types involved in the re-construction of skin tissue are endothelial cells, dermal fibroblasts and keratinocytes. Upon wounding their phenotypes are changed into proliferating and migrating phenotypes infiltrating the wound site. The change in phenotype leading to cell migration occurs in response to gradients of soluble growth factors and insoluble structural ECM molecules, as well as through changes in cell-cell contact (Eckes et al., 2010; Werner et al., 2007). *In vivo*, the different signals always act synergistically, resulting in an optimal control of cell motility. The insoluble ECM components are key signals that initiate cell motility, while growth factors (GF) augment the motility and promote the direction of cell migration (Li et al., 2004). Many cell surface receptors are implicated in cell migration, however, the most important belong to the integrin family, referred to as the “feet” of the cells, as described by Ridley et al. (2003).

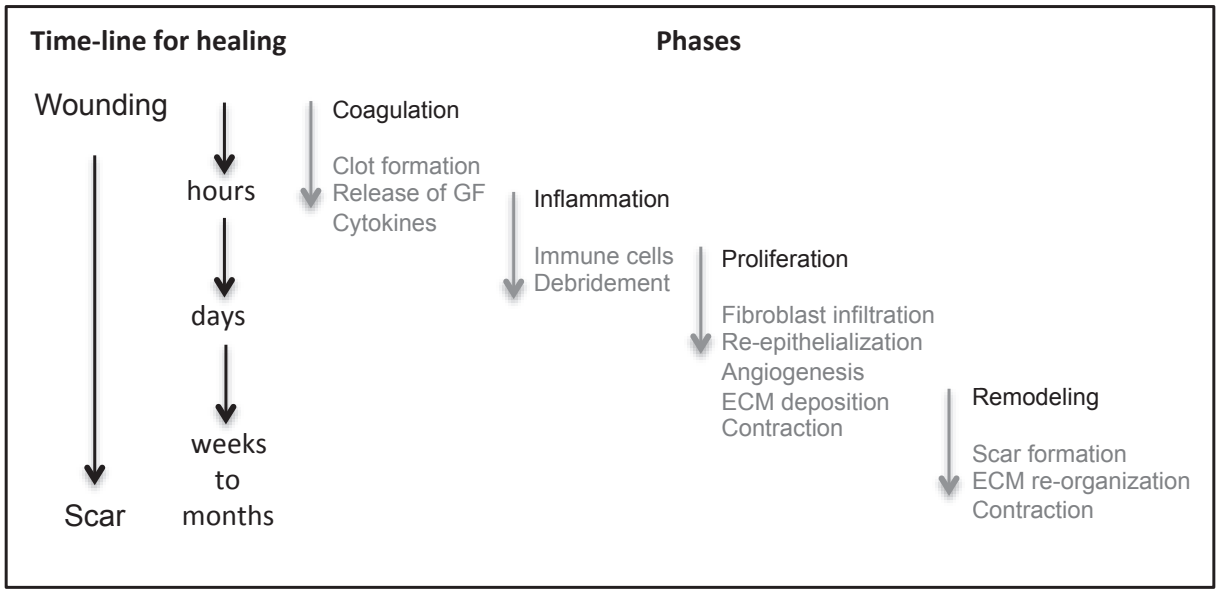


Figure 1. Schematic time-line over the overlapping phases during wound healing; coagulation, inflammation, proliferation and remodeling. Figure modified from Falanga (2005).

Dermal and epidermal wound healing is normally a spontaneous process initiated in the minutes following wounding. However, in larger wounds and non-healing wounds this temporally and spatially controlled process might be impaired, or not sufficient, and therapeutic intervention may become obligatory. For larger wounds, autologous skin grafts are today the treatment of choice (MacNeil, 2007). However, these skin grafts are far from being a universal solution and alternatives are needed. Biomaterials have been investigated for decades for their ability to temporary replace the missing tissue and promote regeneration. Both natural and synthetic materials have shown to be good candidates. To increase the interaction between the, often inert, biomaterials and the surrounding tissue, adhesion- and migration-promoting strategies might be needed.

In vivo, the ECM surrounding all cells is a hydrated network of proteins and proteoglycans. The ECM plays a central role in tissue morphogenesis, homeostasis and repair by providing structural and signaling scaffolds that organize, co-ordinate, and regulate cellular activities (Hynes, 2009). Using selected epitopes of these proteins for specific interaction with skin cells, initiation and regulation of the healing process, is therefore an attractive strategy to functionalize the biomaterials (Lutolf

and Hubbell, 2005). The peptide sequence Arg-Gly-Asp (RGD) might be the best known and investigated ECM derived peptide when it comes to tissue engineering and promotion of cell adhesion (Hersel et al., 2003). Here, we review the use of short RGD-peptides, derived from the ECM, and their use as potential promoters of wound healing by acting on the main cell types of the dermis and the epidermis, the fibroblasts and the keratinocytes, respectively.

2. Biomaterials in dermal and epidermal tissue engineering

Wound dressings and synthetic skin substitutes are used to protect and help the underlying tissue to regenerate (Clark et al., 2007). Wound dressings are placed onto the wound, protect and create a favorable wound environment. However, the wound dressing should be removed from the wound and they are not designed to interact with the tissue. Synthetic skin grafts, on the other hand, are engineered to be a provisional matrix, slowly degrading and progressively replaced by new tissue. The quality of interaction between the cells and these biomaterials are therefore pivotal. Generally, skin substitutes can be divided into two separate groups, cellular or acellular matrices. While the cellular substitutes are seeded with cells *in vitro*, acellular substitutes are colonized *in vivo*. Common for the two groups of skin substitutes is the need for a biocompatible and biodegradable structural backbone, maintaining a moist environment and supporting cell attachment.

In vivo, the cells are surrounded by a polymeric scaffold built from ECM proteins and glycosaminoglycans (GAGs). The properties of the ECM are often mimicked in dermal substitutes by using proteins, polysaccharides, GAGs and synthetic polymers in tissue engineering scaffolds (Table 1). One of the primary roles of the matrix is to keep the wound site moist and under physiological conditions, favoring normal repair. Most polymers or materials used for tissue engineering are able to form polymeric networks retaining large volumes of water, i.e. hydrogels. Hydrogels recreate the gel-like environment of the cells under normal physiological conditions and allows oxygen, nutrients and other metabolites to diffuse into the scaffold (Geckil et al., 2010; Zhu and Marchant, 2011). Moreover, the ECM is also mimicked on a structural level, recreating micro- and nanofibrillar structures of a defined pore-size allowing cell infiltration. Structures with a well-defined organization and pore size

may be prepared through electrospinning (Li et al., 2012; Powell et al., 2008) or by using peptides (Gasiorowski and Collier, 2011; Ohga et al., 2009), proteins or synthetic molecules (Zhou et al., 2009) self-assembling into fibrillar structures. Another approach is to freeze-dry gels to form porous sponges (Ma et al., 2003; Powell et al., 2008; Sang et al., 2011) resulting in structures with a wide range of pore sizes. The strength and flexibility of the matrices were shown to be important for an optimal cell-material interaction and a controlled cell response (Discher et al., 2005; Rehfeldt et al., 2007). Strategies such as covalent crosslinking (Dainiak et al., 2010), polyelectrolytic formation and different ratios of polymers in mixtures (Ouasti et al., 2011; Sun et al., 2011) are a few approaches used to adjust the physical properties of the scaffolds. Moreover, the surface properties, i.e. wettability, hydrophobicity, charge and roughness, of the material were shown to be important for the interaction between the cells and the biomaterial (Bacakova et al., 2011). Another approach to mimic ECM properties is to present insoluble ligands derived from the ECM, such as shorter peptide sequences (Grafahrend et al., 2010; Min et al., 2010) or larger protein fragments, onto the scaffold. In addition to the efforts of increasing the cell interaction with the biomaterial, strategies to induce tissue proliferation and to increase the expression of tissue inducing proteins in the wound have been developed. A common approach to increase proliferation is to add various GFs bound to (Choi et al., 2008), or dispersed in the scaffold matrix (Cai et al., 2005; Hong et al., 2001; Liu et al., 2007). Moreover, transfection of cells through gene delivery is an interesting alternative to induce the expression of tissue-inducing proteins in cells (Orsi et al., 2010; Shepard et al., 2010).

2.1. *Proteins*

Many biomaterials used in skin substitutes are components naturally existing in the wound and active during the healing process. Due to their intrinsic biological properties and biocompatibility these materials are advantageous. Collagens are one of the most predominant proteins in the ECM. The most abundant collagens (collagens I, II, III, V and IX) form fibrils providing a strong, flexible network surrounding the cells and linking the epidermis to the dermis. In addition to strengthening the tissue, the collagen also provides cues for attachment, proliferation and differentiation. Collagen as the base scaffold of dermal substitutes has been well investigated (Ruszczak, 2003), alone (Helary et al., 2010; Powell et al., 2008), in

combination with polysaccharides (Ma et al., 2003; Sang et al., 2011), or combined with GAGs as in the commercialized skin substitutes Integra® Dermal Regeneration template (Integra LifeScience Corp., Plainsboro, USA) and Apligraf® (Novartis, Basel, Switzerland). The gel-forming gelatin, derived from collagen by heat denaturation, has also been investigated successfully for its use in wound healing (Dainiak et al., 2010; Hong et al., 2001).

2.2 *Glycosaminoglycans (GAGs)*

GAGs are long linear polysaccharides, abundant in the ECM of tissues throughout the body, serving both structural and biological functions (Sugahara et al., 2003; Toole, 2004). The general structure of GAGs is a linear chain composed of hexose and hexamine units. Hyaluronic acid (HA) is a non-sulfated GAG, composed of disaccharide-units of alternating D-glucuronic acid and D-N-acetylglucosamine. HA is one of the major components of the ECM of skin, cartilage and the vitreous humor (Price et al., 2007). During wound healing, HA is naturally expressed, and it has been proposed to have a role in scarless prenatal wound healing (Tammi and Tammi, 2009). Due to its favorable properties in wound healing, HA is often included in wound dressings (Cho et al., 2002). In dermal substitutes, hyaluronan has been investigated both alone (Galassi et al., 2000; Price et al., 2006; Wethers et al., 1994) and in combination with other biomaterials (Kirker et al., 2002; Kutty et al., 2007; Ouasti et al., 2011; Shu et al., 2004b). The majority of GAGs are sulfated, e.g., chondroitin sulfate, heparin and heparan sulfate. Common for the sulfated GAGs are the backbone of hexosamine and uronic acid. The number of sulfate groups and their positions gives the various types of GAGs different biological activities. In the ECM, GAGs have both structural roles, signaling as well as binding and stabilization of GFs (Sugahara et al., 2003). In wound healing, the sulfated GAGs are well represented in combination with other GAGs in wound dressings (Kirker et al., 2002; Liu et al., 2007), or as a co-polymer to collagen and gelatin (Grzesiak et al., 1997; van der Smissen et al., 2011; Wang et al., 2006a) or PEG-diacrylate (Cai et al., 2005) in scaffolds for skin substitution.

2.3 *Polysaccharides*

Other natural polymers investigated for wound healing are the polysaccharides cellulose, chitosan, alginate, dextran and their derivatives. The structure of the

backbone is similar to the GAGs, composed of hexose and hexosamino units. Due to their similarities to GAGs, in addition to their biocompatibility, abundant supply and ease of modification, they are interesting as biomaterials in scaffolds. Chitosan is a deacetylated derivative of the structural component, chitin, a structural component in crustaceans and fungi. Due to its positive NH-groups and ability to agglutinate erythrocytes, it has been shown to have hemostatic properties (Rao and Sharma, 1997) and is used in the hemostatic wound dressing HemCon® Bandage (HemCon Medical Technologies, Inc., Portland, USA). *In vivo* assays have shown an accelerated wound healing of wounds treated with chitosan (Boucard et al., 2007; Park et al., 2009; Takei et al., 2012) and it has been reported that wounds treated with chitosan have a higher flexibility and higher breaking strength of the formed scar (Boucard et al., 2007). Additionally, chitosan was shown to have antimicrobial activities, increasing the chances of a successful healing (Burkatovskaya et al., 2006). The positive charges bind to the negatively charged cell surface of bacteria, impairing the integrity of the membrane and causing leakage (Li et al., 2010). Alginate, an anionic polymer extracted from algae, is composed of D-mannuronate and L-guluronate units with polyelectrolytic properties due to the carboxylic groups (Lee and Mooney, 2012). Alginate has been investigated both as hemostatic dressing (Lee et al., 2009), as well as for skin substitutes (Jeong et al., 2010; Lee et al., 2009). Due to the opposite charges of alginate and chitosan, the two polymers are often used in combination, forming polyelectrolytic complexes (Knill et al., 2004; Murakami et al., 2010). Other polysaccharides tested as biomaterials in wound healing are carboxymethylcellulose (CMC), a cellulose derivative. The absorbing properties of the fibers used in wound dressings absorbing fluid and creating a favorable environment for wound healing Aquacel® (ConvaTec Wound Therapeutics™). Due to its structural properties, CMC has also been investigated as scaffold (Ramli and Wong, 2011). Another biopolymer found in bacteria is dextran, a homopolymer built by D-glucans. Its structure in combination with its ability to form hydrogels makes it suitable as a scaffold for wound healing (Sun et al., 2011; Zhang et al., 2011a).

2.4 *Synthetic polymers*

In addition to natural polymers, synthetic polymers have been investigated for their use in wound healing. Synthetic biomaterials are attractive due to the ability to engineer polymeric chains with a determined length and with tailored properties such as biocompatibility and adhesiveness. The high control over the polymeric properties, the design of synthetic polymers forming hydrogels mimicking the viscoelastic properties of the ECM is easily obtained. Moreover, a reproducible production at a large scale is possible, in comparison to the natural polymers often extracted from living organisms. Furthermore, the degradability of the polymers is easily adjusted by inserting groups degraded in the *in vivo* environment, such as hydrolysable moieties or specific sequences sensitive to proteases (Patterson and Hubbell, 2010; Zhu, 2010). Polymers such as polyethylene glycol (PEG; (DeLong et al., 2005a; Kutty et al., 2007; Ouasti et al., 2011), polyvinyl alcohol (PVA; (Schmedlen et al., 2002), poly(lactic glycolic acid) (PLGA; (Grafahrend et al., 2010) and polycaprolactone (PCL; (Choi et al., 2008; Grafahrend et al., 2008; Li et al., 2012) are a few examples on synthetic polymers tested and reported as suitable components in skin substitutes, alone or as block polymers. Moreover, polymerizing synthetic peptides (Gasiorowski and Collier, 2011) and self-assembling peptide-derivatives (Zhou et al., 2009) have also been used to form polymeric networks. However, due to their inertness, synthetic polymers need to be combined with natural biopolymers or functionalized with peptides, proteins, GFs or GAGs to confer biofunctionality to the matrix.

Biomaterials tested for components of skin substitutes			
Classes	**Polymer**	**Co-polymer**	**References**
Proteins			
	Collagen	-	(Powell et al., 2008)
	Collagen	Chitosan	(Ma et al., 2003)
	Collagen	Alginate	(Sang et al., 2011)
	Gelatin	-	(Dainiak et al., 2010)
Glycosaminoglycans			
	Hyaluronic acid	-	(Galassi et al., 2000)
	Hyaluronic acid	PEG	(Kutty et al., 2007)
	Sulfated GAGs	Hyaluronic acid	(Kirker et al., 2002)
		Heparin	(Liu et al., 2007)
		Collagen	(van der Smissen et al., 2011)
		PEG	(Cai et al., 2005)
Polysaccharides			
	Chitosan	Collagen	(Ma et al., 2003)
	Chitosan	Dextran	(Zhang et al., 2011)
	Alginate	-	(Lee et al., 2009)
	Alginate	Collagen	(Sang et al., 2011)
	CMC	-	(Ramli and Wong, 2011)
	Dextran	-	(Sun et al., 2011)
	Dextran	Chitosan	(Zhang et al., 2011)
Synthetic polymers			
	PEG	-	(DeLong et al., 2005)
	PEG	Hyaluronic acid	(Ouasti et al., 2011)
	PVA	-	(Schmedlen et al., 2002)
	PLGA	Polycaprolactone	(Grafahrend et al., 2008)
	Polycaprolactone	-	(Choi et al., 2008)

Table 1. Biomaterials and combinations thereof used for scaffolds in skin tissue engineering. Four classes are represented; proteins, glycosaminoglycans, polysaccharides and synthetic polymers. Abbreviations: polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly (lactic-co-glycolic acid) (PLGA).

3. Arg-Gly-Asp (RGD) and integrins

During the proliferation step in wound healing, fibronectin has a pivotal role for the migration and infiltration of dermal and epidermal cells. FN is deposited on the wound surface (Grinnell et al., 1981) and together with fibrin, FN forms a provisional matrix guiding the fibroblasts to infiltrate and the keratinocytes to migrate during the re-epithelisation step of wound healing (Clark et al., 1982). FN is a glycoprotein found in plasma, in the ECM and on cell surfaces. In the ECM, FN assembles into an insoluble fibrillar network on the cell surface (Mao and Schwarzbauer, 2005). It is a mosaic protein composed of primarily three types of repeating amino acid motifs, type I, II and III domains of ~ 40, 60 and 90 amino acids, respectively (Petersen et al., 1983).

FN has been shown to induce adhesion and migration in many different cell types, including NHK and HDF, through the interaction with the cell surface receptors called integrins. It was discovered that the cells interact with FN through distinct cell-binding domains (Pierschbacher et al., 1981). The main sequence implicated in the contact with cells is the tri-peptide sequence Arg-Gly-Asp (RGD) in module III_{10} (Pierschbacher and Ruoslahti, 1984). In the native conformation of FN, the RGD sequence is extended by about 10 Å away from the molecular body by a loop (Leahy et al., 1996). Being located on the tip of the loop gives the tri-peptide a particular accessibility, ensuring an optimal interaction between the cell receptors and the protein (Fig. 2). The RGD-sequence is not unique to FN but on the contrary present in several other ECM molecules, e.g., laminin and vitronectin. The difference with regard to the activity of these proteins is the conformational exposure of the sequence in the protein. However, RGD is not the only sequence responsible for cell-protein interactions. The Phe-His-Ser-Arg-Asn (PHSRN) sequence (Aota et al., 1994) in III_9 works in synergy with the RGD-sequence (Obara et al., 1988). The low rotation between III_9 and III_{10} places the RGD-loop and the synergy site on the same side of the FN molecule, separated by a distance of 10-30 Å, short enough for a single integrin to span and simultaneously interact with both the RGD motif and the synergy site PHSRN, increasing the signal from FN in the interaction with certain integrins.

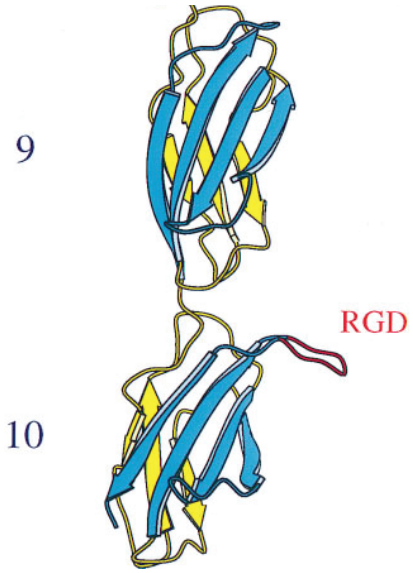


Figure 2. The ternary structure of the type III domains 9 and 10 of fibronectin. In module 10 the RGD sequence is placed on a tip of a loop (Leahy 1996).

Integrins constitute an important class of cell adhesion receptors responsible for the interaction with the ECM, including FN and the RGD sequence. Integrins are transmembrane glycoprotein cell-surface receptors (Hynes, 1987) able to mediate both cell-cell and cell-ECM adhesion and bi-directional signalling. Being αβ-heterodimers, different combinations of subunits determine the specificity of the receptor.

Hitherto, 24 integrins have been identified in humans and several of them have shown to use RGD as their ligand. The need of the PHSRN synergy site for an optimal interaction differs between the various integrins depending on their structure. As an example, αVβ3 has been shown to be independent of the presence of PHSRN, whereas for the α5β1 integrin PHSRN is needed for optimal ligand-receptor interaction (Petrie et al., 2006). Binding between integrins and the ECM is influencing many different responses such as proliferation, cell survival, transcription, cytoskeletal organisation and migration, through numerous pathways (Hynes, 2002). In integrin-mediated adhesion, the integrins form complexes linking the ECM to the cytoskeleton via the integrins, cytoskeletal proteins and a number of enzymes. These complexes are called focal contacts or focal adhesions and include over 50 proteins (Zamir and Geiger, 2001). In integrin-mediated cell adhesion and migration, binding between the ECM and the integrins activates complex signalling cascades

including Rho GTPases, phosphatases and kinases, leading to a re-arrangement of the cytoskeleton and a morphological change of the cell (DeMali et al., 2003; Huveneers and Danen, 2009). The Rho GTPases, Rac and Cdc42 are activated during the early phase of adhesion, regulating filopodia- and lamellipodia-formation through actin polymerization.

In tissue engineering, the use of RGD-containing peptides is an interesting strategy to increase the interaction between the biomaterial and the surrounding tissue (Hersel et al., 2003). Many different cell types respond to exposed RGD-moieties and the peptide has been investigated in tissue engineering for many tissues, e.g., blood vessels (DeLong et al., 2005b; Sundararaghavan and Burdick, 2011), cartilage (Jeschke et al., 2002), bone (Lieb et al., 2005; Zhang et al., 2011b), cardiac tissue (Shachar et al., 2011) and skin (Grzesiak et al., 1997; Shu et al., 2004a). Apart from its role as cell adhesion promoter, a number of different applications have been investigated for RGD-containing peptides. In tumor tissue, certain integrins, such as αVβ3, are overexpressed. Hence, RGD has been used to target tumors by its integrins for the delivery of anti-cancer treatments (Eldar-Boock et al., 2011; Yonenaga et al., 2011) and for imaging (Sugahara et al., 2009). The expression of RGD-recognizing integrins in immune cells has also been used to target these cells and enhance uptake of RGD-functionalized vectors (Brandhonneur et al., 2009; Ouasti et al., 2012). Moreover, the integrin receptor αIIbβ3 plays an essential role in platelet aggregation and RGD-mimicking drugs are used as agonists, inhibiting the binding between fibrinogen and platelets, subsequently inhibiting aggregation and thrombosis formation (Starnes et al., 2011). Today, there are two anti-thrombotic drugs on the market, the non-peptide Aggrastat® (tirofiban; Iroko Cardio International Sàrl, Geneva, Switzerland) and the KGD-containing heptapeptide, Integrilin® (eptifabitide; GlaxoSmithKline, Brentford, UK).

4. Evaluation of RGD-biomaterials

During dermal wound healing, the major cell types involved in re-building the cutaneous barrier are keratinocytes, fibroblasts and endothelial cells, restoring the epidermis, dermis and the blood vessels, respectively. The fibroblasts restore the dermal layer of the dermis together with the endothelial cells forming the blood

vessels. The keratinocytes cover the wound and form the outer barrier, the epidermis. In the following, the interaction between keratinocytes and fibroblasts with RGD-modified biomaterials with the aim of promoting wound healing is reviewed. Endothelial cells have shown to respond to RGD peptides, and have mainly been investigated on vascular grafts (Zheng et al., 2012). This interaction will, however, not be reviewed here.

For keratinocytes the most common way of investigating the *in vitro* effect is to use primary, human keratinocytes (Cooper et al., 1990; Grzesiak et al., 1997) or alternatively to apply the immortalized cell line, HaCaT (Grafahrend et al., 2010; Salber et al., 2007), a well-established cell model for normal keratinocytes (Boukamp et al., 1988). As for fibroblasts, human dermal fibroblasts (DeLong et al., 2005a; Lee et al., 2010), dermal fibroblasts from rats (Monteiro et al., 2011) and cell lines such as NIH 3T3 (Shu et al., 2004a) and L929 (Karakecili et al., 2007), both from murine origin, have been used to evaluate biomaterials *in vitro*. *In vitro* cellular responses such as initial cell adhesion, migration, proliferation and cell morphology are important parameters to evaluate the effect of the biomaterial on the wound healing.

Evaluating the effects *in vivo* of RGD-biomaterials, both human (Hansbrough et al., 1995; Steed et al., 1995) and animal models such as pigs (Mertz et al., 1996), mice (Cooper et al., 1996) and rats (Shu et al., 2004a; Waldeck et al., 2007) have been applied. Pigs are suggested the ideal animal wound healing model with respect to the structure of the skin and the mechanisms of wound healing (Ansell et al., 2012). The most common way of analysing the biomaterial effect on wounds *in vivo* is to measure the wound size before and after the assay. Other methods of analysis are measuring the thickness of the epithelial layer or assessing the number of fibroblasts in the applied matrix.

4.1 *Fibroblasts in vitro*

During the earliest phase of wound healing, the wound is plugged with a clot of platelets embedded in fibrin, fibronectin, vitronectin and thrombospondin (Margadant et al., 2010). The activated platelets and the immune cells secrete cytokines and growth factors, stimulating the infiltration of fibroblasts and endothelial cells into the clot, forming the granulation tissue. In the granulation tissue, the fibroblasts are activated and differentiate into myofibroblasts through external stimuli (Eckes et al.,

2010). The main functions of these contractile fibroblasts are to remodel the granulation tissue through producing new ECM and to contract the wound. Once the remodelling of the new scar tissue is finalised, the myofibroblasts undergo apoptosis and leave a fibrous matrix. The infiltration of fibroblasts into the granulation tissue is an important step to reconstruct the new tissue and form the provisional matrix for re-epithelisation. During the migration, the expression of integrins is up-regulated. Dermal fibroblasts express a wide range of integrins, including the RGD-binding $\alpha V\beta 3$ receptors (Palaiologou et al., 2001). The interaction between dermal fibroblasts and $\alpha V\beta 3$ has been widely investigated and fibroblasts are known to adhere and spread when placed on RGD-substrates (Brandley and Schnaar, 1988; Massia and Hubbell, 1990). The spatial distance of 440 nm in between the RGD-moieties were shown to be optimal to induce spreading (Massia and Hubell 1991).

In tissue engineering, scaffolds are used to fill the space of missing tissue and support the infiltration of cells regenerating new living tissue. Functionalizing scaffolds with RGD is an approach to promote the colonization by adding adhesive moieties. Several different materials, synthetic or of natural origin, have been used in combination with RGD, among them collagen (Monteiro et al., 2011), gelatin (Ito et al., 2003), hyaluronic acid (Park et al., 2003; Shu et al., 2004a), alginate (Lee et al., 2010), chitosan (Karakecili et al., 2007) and synthetic polymers such as PEG (DeLong et al., 2005a), PVA (Schmedlen et al., 2002) and Fmoc (Zhou et al., 2009), to mention a few.

The interactions between dermal fibroblasts and functionalized scaffolds have been investigated for their impact on the level of adhesion, migration, proliferation and morphology of the cells. *In vitro*, a clear increase in the level of cell adhesion on adding RGD-moieties to a biomaterial has been shown (Park et al., 2003). In addition to adhesion, the cells show spread morphology when seeded onto or in an RGD-functionalized biomaterial (Ohga et al., 2009; Ouasti et al., 2011), a larger coverage area (Schmedlen et al., 2002) or show a higher percentage of fully spread cells (Shu et al., 2004a). Regarding the migration, RGD has generally been reported to increase migration and infiltration of fibroblasts in and over biomaterials (Shepard et al., 2010). Additionally, immobilization of RGDS-gradients can direct alignment and migration in PEG-hydrogels along increasing densities of adhesion peptide (DeLong et al., 2005a), which is an interesting feature for the control of cell infiltration

into biomaterials. RGD-functionalized biomaterials have also shown to favor the proliferation of fibroblasts (Park et al., 2003; Shu et al., 2004a).

Overall, RGD-functionalized biomaterials show promising properties to increase the fibroblast-biomaterial interaction *in vitro*. Recent publications on RGD-functionalized biomaterials focused on RGD in combination with other parameters influencing the function of biomaterials, such as supramolecular structures and the mechanical properties of the matrix. Mimicking the formation of fibrillar structures in the ECM, using self-assembling peptides functionalized with RGD-peptides have been shown to be a successful approach modifying the composition and strength of the biomaterial used as a scaffold and additionally offering a spatial control of the orientation of the adhesion peptides. RGD-peptides bound to laminin derived peptides (Ohga et al., 2009), de novo peptides (Collier and Messersmith, 2003; Gasiorowski and Collier, 2011) and Fmoc-groups (Zhou et al., 2009) have all been shown to assemble into fibrillar structures by forming β-sheets. Another approach applied in combination with RGD-mediated adhesion is to control the elastic properties/modulus of biomaterials. By varying the concentration and molecular weight of HA in a cross-linked PEG-diacrylate hydrogel, Ouasti et al. (2011) demonstrated the possibility to increase the RGD-dependent adhesion of fibroblasts, by increasing the modulus of the gel.

Moreover, functionalizing hydrogels with RGD-moieties has been shown to enhance gene delivery in the fibroblastic tissue (Shepard et al., 2010). Transfecting cells is an attractive strategy to confer cells the function of a “bioreactor” for the expression of tissue inductive proteins promoting normal tissue repair and regeneration. Increased densities of RGD in a PEG-gel gave an increased cell migration and subsequently a higher degree of transfected cells. The increase of transfection of migrating cells was suggested to be due to an increased number of encounters with DNA-vectors entrapped in the gel, as well as a favored uptake by cells of a migratory phenotype (Gojgini et al., 2011; Shepard et al., 2010).

4.2 *Keratinocytes in vitro*

During re-epithelisation, keratinocytes at the wound edges and in the hair follicles proliferate and start to migrate in over the wound (Ortonne et al., 1981). Stationary basal keratinocytes are attached to the basement membrane composed of laminin

isoforms and collagen IV and VII. After wounding, this membrane is disrupted and the cells are in contact with additional proteins, such as fibronectin, vitronectin and fibrin, as well as collagens I, III and VI (O'Toole, 2001). During the re-epithelisation step in wound healing, FN is deposited on the wound surface (Grinnell et al., 1981) and the keratinocytes migrate on a granulation tissue rich in fibronectin (Clark et al., 1982). Due to their lack of receptors interacting with fibrin and fibrinogen, the keratinocytes line the wound bed dissecting the clot from the wound bed (Kubo et al., 2001).

The role of biomaterials favoring epithelialization is to form a substratum to the newly formed epidermal layer. In the early nineties, mixtures of collagen and chondroitin sulfate functionalized with RGD-peptides were reported to induce an increased cell attachment (Cooper et al., 1990). RGD-peptides were added to a matrix of collagen I and chondroitin-6-sulfate and inoculated with human dermal fibroblasts and keratinocytes *in vitro*. After four days in culture, a significantly thicker epithelial cell layer was observed on the matrix containing RGD peptides compared to the matrix without peptides. This effect was observed with and without colonization of fibroblasts in the matrix. A similar collagen I and chondroitin-6-sulfate skin substitute (Integra™ Artificial Skin Dermal Regeneration Template™) grafted with longer RGD peptides was used by Grzesiak et al. (1997). The RGD-functionalized matrix was investigated for the initial attachment and spreading of fibroblasts, keratinocytes and endothelial cells. An increased attachment and spreading, specific to the RGD-moieties, of keratinocytes and endothelial cells were reported for an RGD-functionalized matrix compared to the matrix alone.

Moreover, a hyaluronan matrix functionalized with RGD, Argidene™ (Telios Pharmaceuticals Inc., San Diego, CA, USA), was tested *in vitro* by measuring the migration from a porcine explant out over the matrix. Compared to the hyaluronan alone, the RGD-functionalized matrix showed a significantly higher level in outgrowth from the explant compared with the control (Mertz et al., 1996). Other *in vitro* observations of interaction between RGD-peptides and keratinocytes were reported by (Kim et al., 1992), who showed a dose-dependent cell migration on an RGD-substrate. Moreover, RGD-grafted fusion proteins were also shown to increase cell adhesion of keratinocytes (Wang et al., 2006b).

Despite the large number of integrins recognizing RGD as ligands in keratinocytes, few reports on a favorable RGD-keratinocyte interaction have been published. Additionally, simpler models, using synthetic scaffolds and only keratinocytes, RGD fail to induce adhesion and spreading, indicating a lack of receptor expression. Grafahrend et al. (2010) investigated electrospun fibers of a synthetic polymer blend, functionalized with ECM-peptides, as scaffolds for epithelial tissue engineering. The electrospun backbone of ethylene oxide, propylene oxide and poly(lactide-co-glycolide) (PLGA) was functionalized with two different ECM peptides, RGD and GEFYFDLRLKGDK, a sequence from collagen IV. *In vitro* assays showed no interaction between the HaCaT cells and the fibers functionalized with only RGD-sequences after either 1 or 6 days. The collagen peptide, on the other hand, showed both spreading and increased adhesion of HaCaT cells. Similar observations, where HaCaT failed to interact with RGD-moieties, have been reported (Roessler et al., 2001; Salber et al., 2007).

Important parameters differing between the publications reporting positive and negative results are the intrinsic bioactivity of the chosen biomaterial, the cell type, different analysis methods, the time lines for evaluation and the length of the RGD-peptides. All the mentioned factors are plausible explanations for the obtained results. However, seen to the low number of publication and the significant difference in the analysis methods, the role of short RGD-peptides in wound healing and on the impact on keratinocyte interaction remain unclear.

Another key factor that must be questioned is whether the expression of RGD-recognizing integrins in keratinocytes is sufficient to induce adhesion and migration. Although keratinocytes express several integrins using RGD as their ligand, i.e. $\alpha5\beta1$, $\alpha8\beta1$,$\alpha9\beta1$, $\alpha V\beta1$, $\alpha V\beta5$, $\alpha V\beta6$ and $\alpha V\beta8$ (Margadant et al., 2010), the level of up-regulation is for many receptors uncertain. Additionally, the main integrin responsible for cell migration in keratinocytes is $\alpha5\beta1$, and even though $\alpha5\beta1$ recognizes RGD, the integrin requires the synergy site PHSRN in module type III_9 in fibronectin for effective ligand-receptor interaction (Aota et al., 1994; Obara et al., 1988). Keratinocytes expressing mainly $\alpha5\beta1$ are therefore more prone to interact with fragments of fibronectin encompassing the modules type III_9 and III_{10} than with the short sequence RGD (Petrie et al., 2006). Hence, the short RGD-sequence might not be sufficient to induce a change of phenotype alone. More complex approaches

have shown to clearly induce adhesion and migration in keratinocytes (Sharma et al., 2006) and other epithelial cells (Brown et al., 2011). Most reports on increased adhesion, migration and increased thickness of the epithelial layer, have in common the combination of RGD-peptides with a biomaterial with an intrinsic bioactivity, e.g., collagen, chondroitin sulfate and hyaluronic acid. The possibility of an intracellular cross-talk, strengthening the cellular response to RGD, might be an explanation for the difference in cell responses reported. This hypothesis is in line with findings of synergistically increased levels of cell adhesion in HaCaT cells when mixing RGD with a collagen peptide (Grafahrend et al., 2010) and laminin peptides (Salber et al., 2007). These results are supported by the results of (Reyes et al., 2008), showing the synergistic effect, of mixing collagen and the III_7-III_{10} FN fragment, ECM ligands of different receptor affinities on both adhesion level as well as FAK (focal adhesion kinase) activation. The results of Grafahrend et al. (2010) and Salber et al. (2007) confirm the presence of some kind of interaction between RGD and HaCaT cells and also show a simple way of forming more complex biomaterials.

In conclusion, there are contradictory reports regarding the effects of RGD on keratinocytes. Additionally, the low number of reports suggests that RGD alone is not sufficient to induce adhesion and spreading in keratinocytes, but might play an interesting role in more complex strategies.

Biomaterials with RGD-containing peptides tested *in vitro*				
Cell type	**Biomaterial**	**Co-polymer**	**Cell response**	**References**
Fibroblasts	Collagen	-	Promoted adhesion	(Monteiro et al., 2011)
	Gelatin	-	Increased proliferation	(Ito et al., 2003)
	Hyaluronic acid	PEG	Enhanced spreading, attachment and proliferation	(Shu et al., 2004)
	Chondroitin sulfate	Collagen	Increased infiltration of cells into matrix	(Cooper et al., 1990)
	Alginate	-	Spreading of cells and enhanced cell growth	(Lee et al., 2010)
	Chitosan	-	Triggered spreading, attachment and proliferation	(Karakecili et al., 2007)
	PEG	-	Inducing cell migration and infiltration	(Shepard et al., 2010)
	PVA	-	Higher level of adhesion and spreading of cells	(Schmedlen et al., 2002)
	Fmoc	-	Increased spreading and proliferation	(Zhou et al., 2009)
Keratinocytes	Chondroitin sulfate	Collagen	Thicker epithelial layer	(Cooper et al., 1990)
	Collagen	Condroitin sulfate	Inducing attachment and spreading of cells	(Grzesiak et al., 1997)
	Hyaluronic acid	-	Increased outgrowth and migration of cells from explant	(Mertz et al., 1996)
	PLGA	Ethylene/ propylene oxide	No cell adhesion observed	(Grafahrend et al., 2010)
	Star PEG	-	No cell adhesion observed	(Salber et al., 2007)

Table 2. Biomaterials functionalized with RGD-containing peptides and the cell response of dermal fibroblasts and keratinocytes *in vitro*. Abbreviations: polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly (lactic-co-glycolic acid) (PLGA).

4.3. *RGD-biomaterials in vivo*

In the middle 1990's, a hyaluronan matrix functionalized with RGD, Argidene™ (Telios Pharmaceuticals Inc., San Diego, CA, USA) was investigated *in vivo* to accelerate wound healing. Argidene™ was evaluated in a number of different skin lesions in humans. When tested on sickle-cell leg ulcers, a significant accelerated healing rate of the epithelialization was reported (Wethers et al., 1994). In a second study, the RGD-hyaluronan conjugate was investigated for the treatment of diabetic ulcers in a clinical study (Steed et al., 1995). The closure of diabetic ulcers was studied over a time period of one year and showed a four-fold higher number of patients with complete healing than the control group. However, for both studies, a control of saline solution was used. The positive effect of applying a hydrogel on the healing process cannot be excluded to have contributed partly or mainly to the accelerated healing. Moreover, Argidene™ was applied on burns in pediatric patients and compared to the standard treatment, a cream of silver sulfadiazine (Hansbrough et al., 1995). Also here, the wounds treated with Argidene™ showed an accelerated healing and a higher degree of wound closure compared to the control.

Argidene™ was also tested *in vitro* and *in vivo* in a porcine wound model (Mertz et al., 1996). *In vitro*, it was found that the presence of RGD-peptides significantly increased the epithelial migration when studying the epithelial outgrowth from tissue explants after 2-6 days. The migration was shown to be dose-dependent, in agreement with the *in vitro* results presented by Kim et al. (1992). Moreover, the RGD-hyaluronan matrix was investigated for its effects on the epithelialization in mice by using "meshed skin graft interstices" covered with the matrix (Cooper et al., 1996). The epithelialization from the edges of the skin graft was assessed and the two different matrices were compared. Significant increase in wound closure when RGD-hyaluronic acid matrix was used was reported, compared to only hyaluronic acid. Also here the epithelium was reported to be thicker in the wounds treated with the RGD-hyaluronic acid matrix than with the hyaluronic acid alone. *In vivo*, healing was shown to benefit from the RGD-peptides by a faster epithelialization of the wounds and also a thicker epithelial layer

Despite several reports with encouraging results, Argidene™ never reached the market due to larger clinical studies failing to show any significant increase in healing (Ratcliffe, 2011). No further publications can be found on RGD-hyaluronan.

The specific interaction between fibroblasts and hydrogels of a thiolated hyaluronate derivative conjugated with PEG-diacrylate and RGD-peptides was evaluated *in vitro* and *in vivo* in a rat model (Shu et al., 2004a). *In vitro*, RGD increased cell proliferation when seeded onto the surface of the gels, however, when dispersed in the gel, no difference was observed. For the *in vivo* assays, fibroblasts were mixed with gels in the absence and presence of RGD, respectively, and injected subcutaneously. Initially, a more uniform organization of the fibrous tissue and of procollagen was found in the wounds treated with the RGD-containing gels, but at a later time-point no difference was observed between the two gels. Waldeck et al. (2007) investigated the effect of a gelatin gel modified with PEGylated RGD-peptides and soluble keratinocyte growth factor-1 (KGF-1) in dermal wound healing in a rat model. The wound treated with the gel had a faster inflammatory response and higher level of tissue organization compared to the control gel without KGF-1 and RGD. However, no difference in cell number was found when comparing the two systems, questioning the role of RGD in *in vivo* applications for enhanced adhesion and migration of fibroblasts.

Overall, promising studies on the *in vivo* effect on fibroblasts and keratinocytes in wound healing have been published. Despite encouraging results, none of the matrices reached the market and no further development of the products can be found. Recent reports investigating the role of RGD favoring wound healing *in vivo* show no clear enhancement of cell infiltration or tissue formation for either keratinocytes or fibroblasts and hence no clear role of RGD can be deduced from these reports.

5. Conclusion

To summarize, RGD has promising properties to confer functionality to biomaterials for improved cell-biomaterial interactions. For fibroblasts, RGD clearly confers attractive features to biomaterials for fibroblasts such as increased adhesion, spreading and migration *in vitro*. The interaction between keratinocytes and RGD *in*

vitro is less documented and both positive and negative results have been reported. The variation in the results suggests a more complex interaction between RGD and keratinocytes and warrant further investigation.

During the nineties, a series of positive reports were published, stating a promoted wound healing *in vitro* and *in vivo*, however, no continuation can be found either in publications or in the form of commercialized products. In recent publications no significant efficacy can be seen *in vivo*. The lack of reports showing an enhanced infiltration or promoted wound healing *in vivo*, suggests that the general idea of adding RGD to confer functionality to a biomaterial is to simplistic for successful *in vivo* use. RGD alone might be insufficient to induce and promote wound healing, however, *in vivo* it might play an essential role as a part of a more complex approach in strategies promoting wound healing.

New approaches, such as mixing adhesion peptides, new combinations of biomaterials and RGD, or an alternative nanoscaled exposure of RGD-peptides might be an approach to confer more complex signalling and to mimic intracellular crosstalk present *in vivo* in order to obtain stronger response for future biomaterials.

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Chapter II:

In vitro Evaluation of an RGD-Functionalized Chitosan Derivative for Enhanced Cell Adhesion

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Tissue repair is a spontaneous process that is initiated on wounding. However, if this complex mechanism is impaired or not sufficient the use of biomaterials might increase the chance of successful healing. In this view, an RGD-functionalized polymer was developed to promote dermal healing. A water-soluble chitosan derivative, carboxymethyl-trimethylchitosan (CM-TM-chitosan) was synthesized and GRGDS-moieties were grafted to the backbone at a concentration of 59 nmol/mg polymer to increase cell-biomaterial interaction. Tested *in vitro* with cultured human dermal fibroblasts, the developed polymer showed good biocompatibility and the initial adhesion was increased by 3-5 times due to the GRGDS-moieties. Moreover, cell spreading was specific to the interaction with GRGDS, giving a 12-fold increase of cells showing a fully spread morphology within 30 min. Overall, CM-TM-chitosan conjugated with GRGDS-peptides may prove useful as a biomaterial in wound healing.

Key words: wound healing; human dermal fibroblast; chitosan; RGD peptide; cell adhesion

Abbreviations: trimethyl chitosan (TM-chitosan); carboxymethyl-trimethyl chitosan (CM-TM-chitosan); Arg-Gly-Asp (RGD); Gly-Arg-Gly-Asp-Ser (GRGDS); Ser-Asp-Gly-Arg-Gly (SDGRG)

1. Introduction

In wound healing and tissue engineering, infiltration of cells into and over the wound site is critical. Therefore cell adhesion- and migration-promoting properties of biomaterials are decisive. *In vivo*, cells are surrounded by the extracellular matrix (ECM), a hydrated network of proteins and proteoglycans that provide structure and guidance for the cells in most vital processes (Hynes, 2009). Collagens, and glycoproteins such as laminins and fibronectins are biologically active proteins present in the ECM with important roles in the regulation of cell adhesion and migration. It has been shown that short amino acid sequences in these proteins, interacting with specific cell receptors, are responsible for the induction of cell responses. Examples of peptide sequences shown to be able to induce adhesion in various cell types are YIGSR from laminin (Graf et al., 1987), GFOGER from collagen (Knight et al., 2000) and RGD, first identified in fibronectin (Pierschbacher and Ruoslahti, 1984). Developing hybrid biomaterials equipped with such peptide sequences represents an attractive alternative to confer functionality improving cell-biomaterial interaction.

The amino acid sequence RGD (arginine-glycin-aspartic acid), is a tripeptide found in ECM proteins such as fibronectin, vitronectin and laminin (Hersel et al., 2003). RGD is known to induce adhesion and migration through interaction with several members of the integrin family, e.g., αV-integrins, αIIbβ3, α5β1 and α8β1 (Danen and Sonnenberg, 2003). The binding between RGD and the integrin triggers the Rho GTPase pathway, regulating the rearrangement of the cytoskeleton (Huveneers and Danen, 2009), resulting in the initiation of cell spreading, adhesion and migration.

Due to its reported biocompatibility, biodegradability and its ease of modification, chitosan has been widely investigated for its biomedical and pharmaceutical application (Alves and Mano, 2008; Amidi et al., 2010; Baldrick, 2010; Muzzarelli, 2009, 2011; Muzzarelli et al., 2012). Chitosan is a co-polymer composed of randomly distributed D-glucosamine and N-acetyl-D-glucosamine monomers (Jollès and Muzzarelli, 1999; Muzzarelli and Muzzarelli, 2005). Derivatives of chitosan been synthesized with success, tailoring its properties such as increased water solubility or adding a permanent positive or negative charge, for various applications (Muzzarelli, 1988). Moreover, chitosan has been shown to favor wound healing at several different stages. It was reported to have hemostatic activities through

activation of platelets (Lord et al., 2011) and moreover, an increased infiltration of inflammatory cells and a higher angiogenic activity promoting wound healing, has been observed in wounds treated with chitosan (Boucard et al., 2007; Scherer et al., 2009).

Due to its intrinsic wound healing promoting properties, chitosan and its derivatives have been extensively investigated as a biomaterial for soft tissue repair, alone and in combination with other polysaccharides and glycosaminoglycans (Boucard et al., 2007; Denuziere et al., 1998; Murakami et al., 2010; Takei et al., 2012). During wound healing, the two major cell types, keratinocytes and fibroblasts, proliferate and change their phenotype to the migratory state (Li et al., 2004). They enter the wound site to reconstruct the new tissue. Fibroblasts interact with RGD-sequences, mainly through $\alpha V\beta 3$ integrins (Massia and Hubbell, 1991). Scaffolds and polymeric gels functionalized with RGD-sequences are therefore attractive alternative adhesion/migration-promoting substrates to enhance dermal repair. In this view, we have developed a chitosan derivative functionalized with GRGDS-moieties for use in formulations such as polyelectrolytic complexes, gels and layer-by-layer coatings with the aim to promote wound healing.

2. Materials and methods

2.1 Materials

For synthesis, chitosan (ChitoClear Cg10, 7 and 15 mPa*s) was bought from Primex, (Siglufjordur, Iceland), GRGDS- and SDGRG-peptides from Bachem (Bubendorf, Switzerland), dialysis membranes from Spectra (Breda, The Netherlands) and syringe filters from Millipore AG (Zug, Switzerland). For *in vitro* bioactivity assays, cell culture media and additives were obtained from PAN Biotech GmbH (Aidenbach, Germany) and plastics from Corning (Amsterdam, The Netherlands). The XTT cell proliferation kit 2 was bought from Roche (Basel, Switzerand). For immunofluorescence staining, phalloidin conjugated Alexa 488 was bought from Lonza (Basel, Switzerland), Vectashield mounting medium from Vector Laboratories (Peterborough, UK) and paraformaldehyde from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and reagents were bought from Sigma-Aldrich (Buchs, Switzerland).

2.2 Synthesis of GRGDS-CM-TM-chitosan

2.2.1 Trimethylation of chitosan

Trimethylation of chitosan was achieved through nucleophilic substitution by methyl iodide (MeI) using a modified protocol by Heuking et al. (2009), shown in Fig. 1. Briefly, chitosan was suspended in 1-methyl-2-pyrrolidinone (NMP; 40 ml NMP/g chitosan) and the suspension was kept at pH 10 by addition of NaOH 15%. MeI (6 ml MeI/g chitosan) was added and the mixture let react under a reflux condenser at 60˚C for 100 min. After filtration on a P3 glass filter, the resulting trimethyl-chitosan (TM-chitosan) was precipitated and washed twice with 5 volumes of a mixture of diethyl ether and ethanol (1:1, v/v). The final product was solubilised in 10% NaCl and purified by dialysis using a Spectra/Por 4 dialysis membrane with a molecular weight cut-off of 12-14 kDa over three days before lyophilization.

2.2.2 Carboxymethylation of trimethylchitosan

To obtain CM-TM-chitosan, the freeze-dried TM-chitosan was suspended in NMP (10 ml NMP/100 mg TM-chitosan) at room temperature overnight. The pH was readjusted to a value of 10 with NaOH 15% before adding chloroacetic acid (20 mol equivalents/TM-chitosan sugar unit). The pH was kept constant at a value of 10 during the whole reaction. After 3h the resulting carboxymethyl-trimethyl-chitosan (CM-TM-chitosan) was precipitated in 5 volumes of a mixture of diethyl ether and ethanol (1:1, v/v) and washed twice. The CM-TM-chitosan was solubilized in Milli-Q water and the pH was adjusted to a value of 5 by the addition of 5 N HCl, before being filtered through a 0.45 µm syringe filter. Dialysis was performed as described for TM-chitosan, over three days while changing the water three times per day. The purified product was lyophilised for storage.

2.2.3 Conjugation of GRGDS to carboxymethyl-trimethyl chitosan

In the last step, the GRGDS peptide was grafted to a CM-TM-chitosan backbone via the carboxyl group by a carbodiimide reaction. First, CM-TM-chitosan was dissolved in MilliQ water and the pH was adjusted to neutral. Thereafter, 5 mol equivalents to the carboxyl groups of CM-TM-chitosan of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) each, were added to activate the carboxyl groups of CM-TM-chitosan. The pH was readjusted to a value of 7 and the mixture was left for 1 hour. The GRGDS- and SDGRG-peptide respectively, dissolved in MilliQ-water were added and the reaction was performed

over a time period of 96 h with the pH being maintained at a value of 7. The finalized product was adjusted to pH 3 by adding HCl 1 N and filtered through a 0.45 µm syringe filter. Before being lyophilized for storage, the polymer was purified by dialysis for 3 days. The water was changed three times per day. The total yield of the reaction was 0.67 ± 0.06 (n=3 batches), and the yields of peptide grafting were 0.12 for GRGDS-CM-TM-chitosan and 0.10 for SDGRG-CM-TM-chitosan, as characterized by amino acid analysis (AAA).

2.3 Characterization of polymer

2.3.1 ^{1}H-Nuclear magnetic resonance

^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 300 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). All compounds were dissolved in D_2O or in 0.1 % DCl in D_2O. The samples were analyzed at 25˚C. Chemical shifts (δ) are relative to the internal standard 3-trimethylsilyl propionic acid-d_4 sodium salt (TSP; δ=0.0 ppm for ^{1}H). The shifts were assigned as follows (An et al., 2009; Hjerde et al., 1997): $NCOCH_3$ (δ 2.1), $N(CH_3)_3$ (δ 3.3), NCH_2COOH (δ 3.95), OCH_2COOH (δ 4.35) in position 6 and OCH_2COOH (δ 4.5) in position 3.

The degrees of substitution (DS) for N-acetylation, N-trimethylation and N- and O-carboxymethylation were determined. To estimate the fraction of monomers substituted with N-acetyl groups, trimethyl- and carboxymethyl groups the following equation was used:

$$DS = [substituent\,]/[H]\ \times\ 1/n$$

Where [H] is the integral value of the H-1 peaks between 5-5.6 ppm, [substituent] the integral value of the group and n the number of equivalent protons present in the substituted group.

2.3.2 Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) analysis was performed using a PerkinElmer Spectrum 100 FTIR spectrometer (PerkinElmer, Schwerzenbach, Switzerland). All transmission spectra were recorded in the region of 650 to 4000 cm^{-1}.

2.3.3 Amino acid analysis

The concentrations of peptides in the samples were determined by quantitative amino acid analysis (AAA) using a Biochrom 30 (Biochrom Ltd., Cambridge, UK). Briefly, the samples were hydrolyzed and separated by an ion exchange column. After post-column derivatization by ninhydrin, the samples were analysed at wavelengths of 440 and 570 nm. An internal standard of norleucine was used to determine the concentrations of amino acids in the sample.

2.4 Cell culture

Human dermal fibroblasts (HDF) were a kind donation by Dr. Lee Ann Laurent-Applegate (CHUV, Lausanne, Switzerland). The HDF were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, 10% fetal calf serum and 1% penicillin/streptomycin. The cells were split at sub-confluency with trypsin/EDTA diluted in phosphate buffered saline (PBS). The HDF were used between passages 6 and 10.

2.5 Cytotoxicity

The cytotoxicity of the polymers on HDF was assessed using an XTT-cell proliferation kit 2. Cells were seeded in a 96-well plate at an initial concentration of 10-15 x 10^3 cells/well. After 24 hours the medium was changed to fresh medium containing indicated concentrations of GRGDS- or SDGRG-CM-TM-chitosan. The cells were cultured in the presence of the polymers for 24 hours before removing the medium and adding XTT-reagent. The absorbance was measured at a wavelength of 490 nm after 6 h with a Tecan plate reader (Tecan group Ltd., Männedorf, Switzerland) and the cell viability (%) was expressed in percentage relative to a control group of HDF cultured in the absence of either polymer.

2.6 Bioactivity

2.6.1 Adhesion assay

96-well plates (Costar) were coated with GRGDS- or SDGRG-CM-TM-chitosan at indicated concentrations by overnight adsorption at 4˚C. The wells were subsequently saturated with 1% bovine serum albumin (BSA) for 1h at ambient temperature. The HDF were detached and rinsed in serum-free DMEM before being seeded at a density of 5 x 10^4 cells/well. After 30 min of incubation (37˚C, 5% CO_2), the non-adhered cells were removed by washing with PBS and the attached cells were fixed for 1 hour with a solution of 1% glutaraldehyde in PBS. To evaluate the level of adhesion, the cells were stained for 1 hour with a solution of 0.1 % crystal violet. After drying, the level of adhesion was determined by solubilizing the dye in 1% acetic acid containing 0.1% Triton-X 100 and measuring the optical density at a wavelength of 570 nm with a Tecan plate reader. For competitive inhibition assays, soluble GRGDS- or SDGRG-peptide (1 mM), were added to the cell suspension 15 min prior to seeding. Cell adhesion assays were then performed as described above.

2.6.2 Immunofluorescence staining

Drops of GRGDS- and SDGRG-CM-TM-chitosan solutions (0.1 mg/ml) were placed on cover glasses and let dry. Once dry, they were placed in Costar 24-well plates and blocked by addition of 1% BSA in PBS for 1 hour. Adhesion assays were performed as mentioned in section 2.6.1. The cells were fixed in pre-warmed PBS containing 4% paraformaldehyde, for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 3 min. Samples were then pre-incubated with 1% BSA in PBS for 20 min to prevent non-specific attachment, before being incubated with 6.6 µM phalloidin conjugated to Alexa 488 in 1 % BSA in PBS for 20 min. After being washed in an excess of PBS, the fixed and stained cells were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) before being attached to a microscopic slide. The cell morphology was analyzed with a Zeiss LSM 700 confocal microscope (Carl Zeiss AG, Feldbach, Switzerland) using x10 and x20 objectives.

2.6.3 Spreading analysis

The areas of the spread cells were determined using ImageJ software (available from National Institutes of Health, Bethesda, MD, USA). The surface area of 200-300

cells for each condition and assay were measured. For each condition tested, the mean cell surface was calculated. To analyze the cell morphology, the cells were divided into groups based on their surface. The following size criteria were used; round cells (not spread) <500 μm^2, semi-spread cells 500-1000 μm^2 and spread cells >1000 μm^2. The values were averaged over 5 assays.

2.6.4 Statistical significance

Student's t test (for two samples, assuming equal variance) was used to compare data at a significance level of $p < 0.05$. The results were expressed as mean ± standard deviation.

3. Results and discussion

3.1 Characterization of GRGDS-CM-TM-chitosan

The functionalized chitosan derivative was synthesized by three successive steps: trimethylation of the amine groups, an O- and N-carboxymethylation and in a final step the polymer was functionalized with GRGDS-peptide (Fig. 1). The synthesized polymers, TM-chitosan and CM-TM-chitosan, were characterized with respect to the degree of substitution (DS) and the introduction of the new functional groups by ^{1}H-NMR.

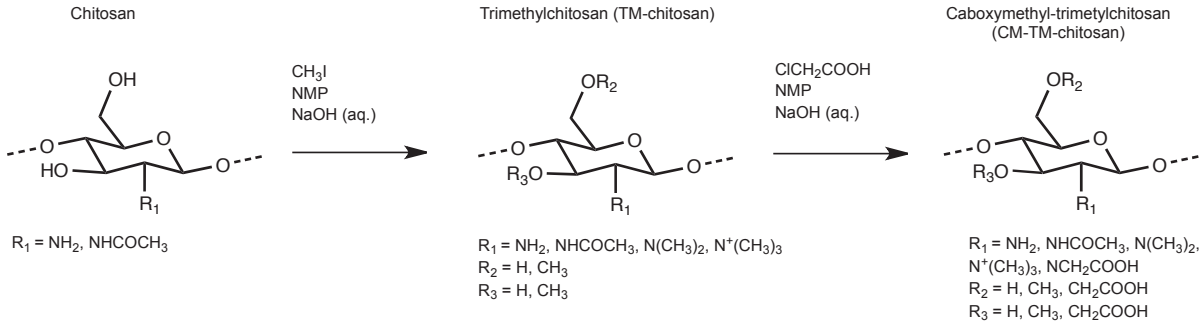


Figure. 1. Synthesis of trimethylchitosan (TM-chitosan), and subsequently carboxymethyl-trimethylchitosan (CM-TM-chitosan), from chitosan in a two-step chemical reaction. Chitosan was trimethylated by a reaction with iodomethyl to form TM-chitosan. TM-chitosan is thereafter carboxymethylated with chloroacetic acid to form CM-TM-chitosan.

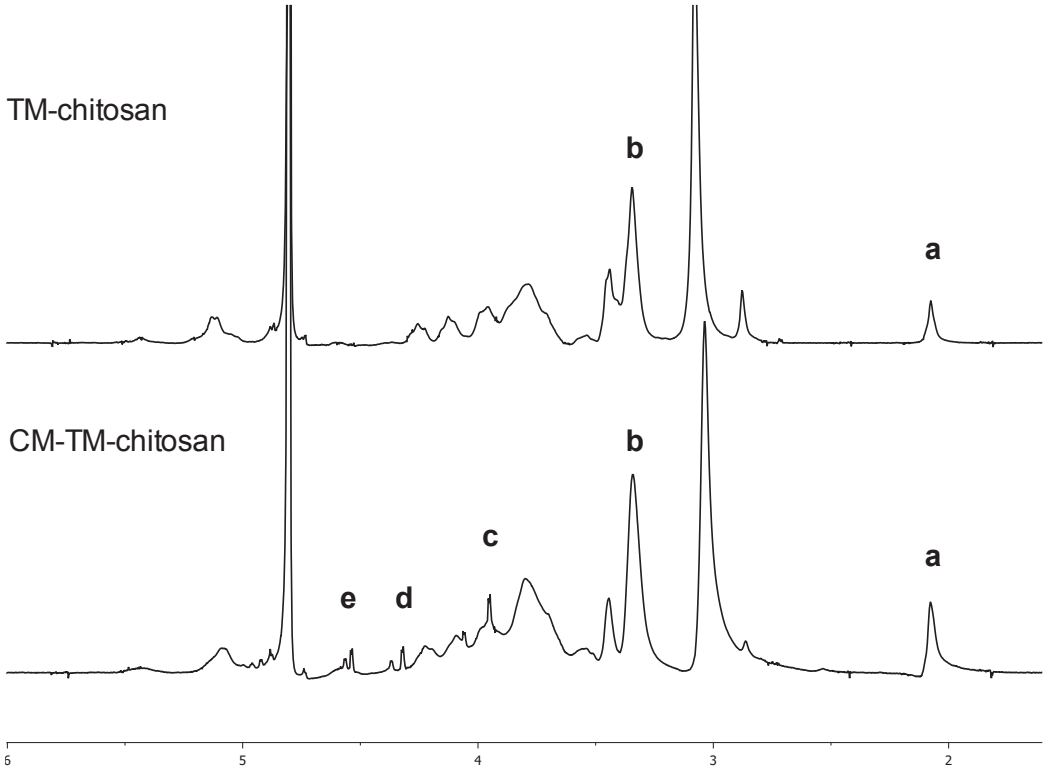


Figure 2. ^{1}H-NMR spectra of TM-chitosan and CM-TM-chitosan. Common for the two spectra are the acetyl- and trimethyl-groups represented by peaks at 2.1 (a) and 3.3 ppm (b), respectively. In the CM-TM-chitosan-spectrum, the peaks at 3.95 (c), 4.35 (d) and 4.55 ppm (e) are corresponding to carboxymethyl-groups in position, 2, 6 and 3, respectively, on the chitosan backbone.

In Fig. 2, the ^{1}H-NMR spectra of TM-chitosan and CM-TM-chitosan are shown. The DS of N-acetylation was determined by the peak at 2.1 ppm in the proton spectra. The degree of acetylation was calculated to be between 0.2 and 0.3, depending on the batch used, values close to those claimed by the provider. The peak at 3.3 ppm (Heuking et al., 2009), present in both spectra, confirmed the N-trimethylation of the chitosan backbone at a DS of 0.4 ± 0.08 (n=3 batches). Similar values were found in the analyses of CM-TM-chitosan. Furthermore, four new peaks appeared in the spectra of CM-TM-chitosan in addition to the functional groups already described for TM-chitosan. Hjerde et al. (1997) reported that the peaks by 3-O- and 6-O-

carboxymethyl groups occurred in the range of 4.1 to 4.6 ppm, and the two peaks at 4.55 and 4.35 were therefore assigned to the carboxymethyl groups in position 3 and 6, respectively. The peak at 3.95 in the CM-TM-chitosan spectra might be assigned to NCH_2COOH (An et al., 2009) and indicated carboxymethylation of the amine in position 2, despite the acetylation and methylation at this position. However, DS in this position is difficult to estimate, since the peak is overlapping with the protons from the carbon ring. Traces of carboxymethyl groups at 4.2 ppm with a carbon resonance at 44.5 ppm (data not shown) were confirmed to be free chloroacetic acid ($ClCH_2COOH$). The total degree of substitution for the carboxymethyl-groups was determined to be 0.15 ± 0.05 in O-6 position and 0.12 ± 0.05 in O-3 position (n=3 batches). Although a higher substitution degree in position O-6 could be expected due to its nature of a secondary alcohol, no significant difference of substitution between the two positions was shown. However, the ratio of substitution in position O-3 and O-6 has shown to change with the water content during the reaction, showing very little or no difference between the positions at certain water/solvent ratio (Chen and Park, 2003), explaining the lack of specificity between the positions.

The results from the NMR-analysis were confirmed by FTIR. Spectra of TM-chitosan, CM-TM-chitosan and GRGDS-CM-TM-chitosan are shown in Figure 3. The absorption band at 1474 cm^{-1} (CH_3 umbrella) confirmed the introduction of methyl groups on the chitosan backbone for TM-chitosan and its derivatives. Furthermore, the insertion of a carboxyl group in CM-TM-chitosan is confirmed by the new peak at 1750 cm^{-1} (C=O stretch, pH of sample 5-6 when solubilized in water) (Chen and Park, 2003; Pretsch et al., 2000). Moreover, the peaks at 1607 cm^{-1} and 1474 cm^{-1} were attributed to asymmetric and symmetric stretch of COO^-, respectively, for the moieties in its carboxylate anionic form bound to Na^+. Finally, in the spectra of GRGDS-CM-TM-chitosan, the introduction of the amide-bond, formed when grafting the peptide to the backbone, is visible as absorption bands at 1641 (C=O stretch) and 1551 cm^{-1} (N-H bend), both typical for an amide bond. In combination with the NMR-analysis, these results showed that we successfully synthesized a new polymer, GRGDS-CM-TM-chitosan.

As a final step of the characterization, the level of substitution of peptide to the chitosan backbone was assessed through amino acid analysis (AAA). Two peptides were used, GRGDS and SDGRG. The latter is an inversed RGD-sequence without

any biological activity and hence used as a control group to GRGDS. The batches were functionalized with 69 nmol/mg and 59 nmol/mg for GRGDS- and SDGRG-CM-TM-chitosan, respectively. Massia and Hubbell (1991) determined the surface RGD-density needed to induce fibroblast spreading to be 10 - 10^4 pmol/cm^2. Provided that all GRGDS-moieties are exposed and active in GRGDS-CM-TM-chitosan in a typical adhesion assay, the peptide concentration corresponds to the given range.

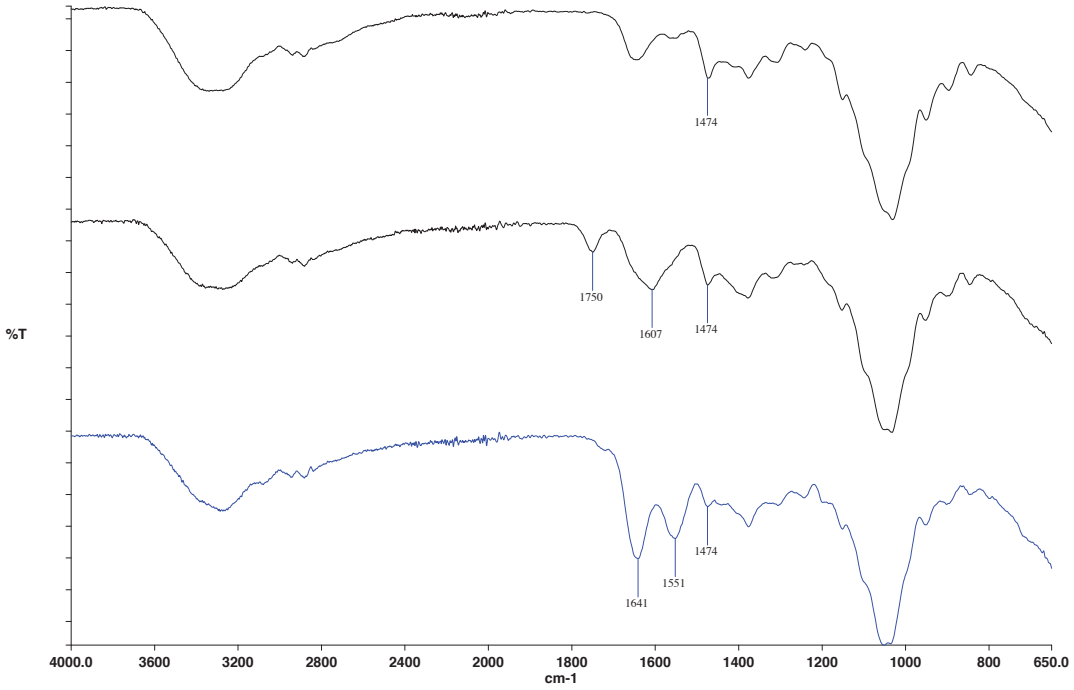


Figure 3. FTIR-spectra of (A) TM-chitosan; (B) CM-TM-chitosan and (C) GRGDS-CM-TM-chitosan.

3.2 Biocompatibility

The biocompatibility of GRGDS-CM-TM-chitosan and SDGRG-CM-TM-chitosan was tested by initial *in vitro* cytotoxicity tests. Human dermal fibroblasts (HDF) were chosen as a suitable cell model for dermal application. As shown in Fig. 4, a slight decrease of the cell viability was observed at increasing concentrations of the polymers. At the maximal tested concentration of 1 mg/ml, the cell viability was reduced to 75 %. However, for the concentrations used in this work no cell viability loss was observed either by GRGDS-CM-TM-chitosan or the control polymer

SDGRG-CM-TM-chitosan. Mimicking the conditions used in adhesion assays, toxicity assays were performed on cells seeded onto coatings at different concentrations of GRGDS-CM-TM-chitosan. After 24 hours of culture, the cell viability was determined at 98 ± 5 % and 95 ± 4 % (n=3 assays) for coatings formed with GRGDS-CM-TM-chitosan of 0.1 and 0.5 mg/ml, respectively. With these results, it was concluded that no adverse effects were shown even after culture on thicker layers of GRGDS-CM-TM-chitosan.

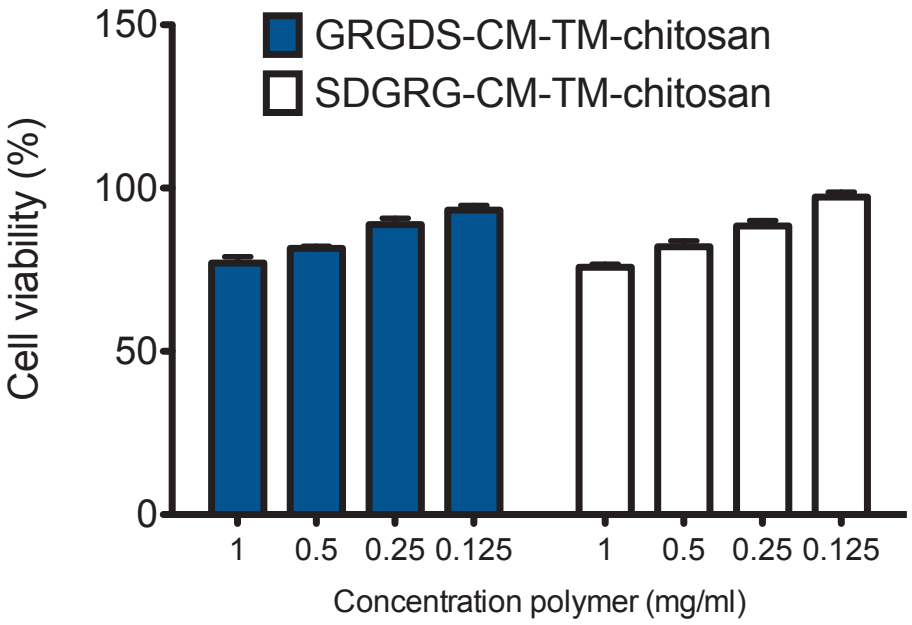


Figure 4. The cell viability of cultured HDF was assessed by XTT-cell proliferation assays after 24 hours in the presence of GRGDS-CM-TM-chitosan and SDGRG-CM-TM-chitosan at indicated concentrations.

3.3 Bioactivity

To assess the functionality of the GRGDS-peptide after grafting, a series of adhesion assays was performed. In a first step, the adhesion of HDF was tested on increasing coatings of GRGDS grafted polymers. HDF attached in a dose-dependent manner to the coatings (data not shown). In a second step, the scrambled sequence of GRGDS was grafted to CM-TM-chitosan.

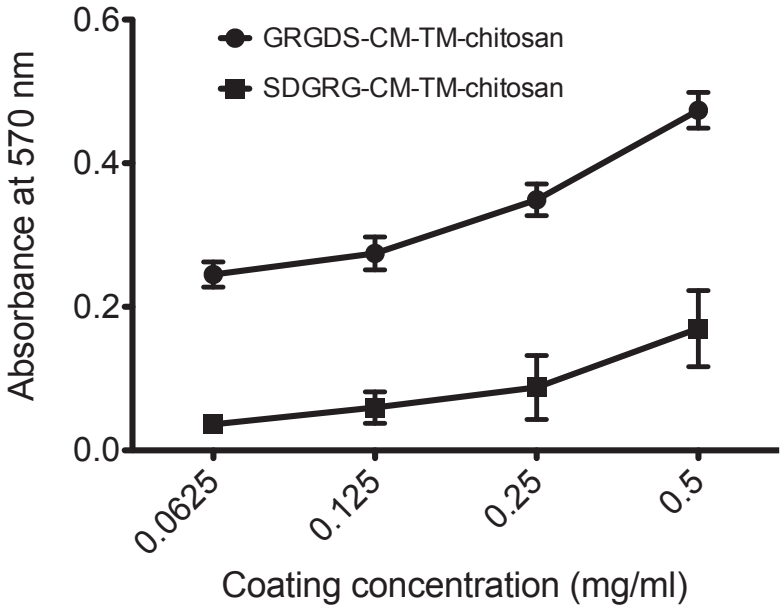


Figure 5. The level of adhesion after adhesion assays onto coatings of a GRGDS- and SDGRG-CM-TM-chitosan of different concentrations assessed by coloration of the cells and measurements of the absorbance. A 3 to 5-fold increase of the adhesion onto the polymer containing GRGDS-peptides compared the polymer grafted with SDGRG was observed.

As shown in Fig 5, the inactive polymer, SDGRG-CM-TM-chitosan, was used as a negative control in the adhesion assays. An increase in adhesion ranging from three-fold for the coating of 0.5 mg/ml, to five-fold for 0.0625 mg/ml was observed in the wells coated with GRGDS-CM-TM-chitosan compared to the SDGRG-CM-TM-chitosan. This demonstrates that the major part of the adhesion is likely to be due to the interaction between integrin receptors and the GRGDS moieties. However, the non-specific adhesion to SDGRG-CM-TM-chitosan is increasing with increasing concentrations of polymer deposited, indicating a second mechanism of interaction between the polymer and cells. Surface properties, such as, polarity, surface charge and surface roughness have been reported to play a role in the attachment of cells to surfaces (Bacakova et al., 2011). Seen the slightly cationic properties of the derivative, and the negative surface charge of the cells, the cell-coating interaction is attributed to be of electrostatic character. This theory is supported by the well-documented mucoadhesive properties of chitosan, and its cationic derivatives, shown to be due to the electrostatic interaction (He et al., 1998; Jintapattanakit et al.,

2008). To conclude, the level of adhesion appears to be determined by at least two factors, with the major parameter being the presence of GRGDS-peptides acting as binding sites for the cells. Secondly, the physico-chemical properties of the polymer, such as charge, influence the degree of adhesion in a non-specific manner.

3.4 Cell morphology

In addition to an increased level of cell attachment to GRGDS-CM-TM-chitosan in comparison to SDGRG-CM-TM-chitosan, the morphology of the cells was remarkably different on the different coatings. In the wells with a polymer presenting GRGDS-peptides, the majority of the cells spread and displayed a flat phenotype, whereas the cells on the coatings with the scrambled peptide were of spherical phenotype. These observations indicated an interaction between the GRGDS-moieties and the integrins, causing a re-arrangement of the cytoskeleton and subsequently spreading of the cell. To further investigate the morphology of the HDF during the initial adhesion, the F-actin and the nuclei of the cells were stained with phalloidin conjugated Alexa 488 (green) and DAPI (blue), respectively, after adhesion assays (Fig. 6).

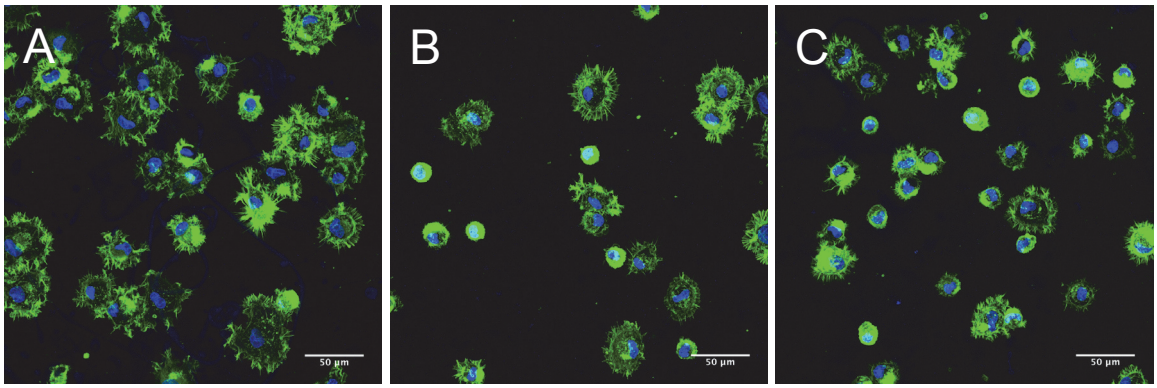


Figure 6**.** Confocal images of human dermal fibroblasts (HDF) stained with phalloidin conjugated Alexa 488 (green) and DAPI (blue) for visualization of cytoskeletal actin-filaments and the nuclei, respectively. The images represent the morphology of the cells 30 min after seeding onto GRGDS-CM-TM-chitosan coating under normal conditions (A) or in the presence of free GRGDS (B) and onto a coating of SDGRG-CM-TM-chitosan (C).

Cells plated on GRGDS-CM-TM-chitosan coatings were compared to cells plated on SDGRG-CM-TM-chitosan. Moreover, cells plated on GRGDS-CM-TM-chitosan under normal conditions and under competitive inhibition conditions were analyzed to confirm the interaction between GRGDS and RGD-dependent integrin receptors. In order to assess the degree of spreading, the mean cell surface area of the cells was determined for the different conditions, as shown in Fig. 7A. The mean surface (n=5 assays) of the cells plated onto GRGDS-CM-TM-chitosan was 1134 ± 224 μm^2, whereas the cells plated onto SDGRG-CM-TM-chitosan had a mean surface of 445 ± 35 μm^2. The significant size difference indicated two distinctly different morphologies of the cells as a result of the coating they were placed on. Furthermore, competitive inhibition was used as a method to show specific RGD-dependent cellular interactions. In the presence of free GRGDS in solution (1 mM), the mean cell surface decreased to 530 ± 98 μm^2 and hence the spreading was successfully inhibited. On the other hand, the presence of free SDGRG (1 mM) did not inhibit cell spreading, proving the specificity of the GRGDS-peptide.

In Figure 7B, the distribution of cell morphology is shown. Depending on their surface area, the cells were divided into three groups. Cells with a surface area of less then 500 μm^2 were marked as non-spread, round cells, cells between 500-1000 μm^2 were marked as semi-spread and cells above 1000 μm^2 as fully spread cells. When plated on GRGDS-CM-TM-chitosan coatings, the majority of cells showed semi- or fully spread phenotypes, whereas only 19 ± 9% of the cells were completely round. For the cells seeded on SDGRG-CM-TM-chitosan, 70 ± 5% of the cells were non-spread and only 4 ± 1% of the cells had a surface area exceeding 1000 μm^2. Furthermore, free GRGDS-peptide added to the media was shown to efficiently inhibit spreading of HDF by competitive binding. In the presence of GRGDS, the number of fully spread cells decreased significantly from 52 ± 14% under normal conditions, to only 8 ± 5% ($p < 0.0002$). The number of round cells increased significantly to 60 ± 15% compared to 19 ± 9 % under normal conditions ($p < 0.0003$).

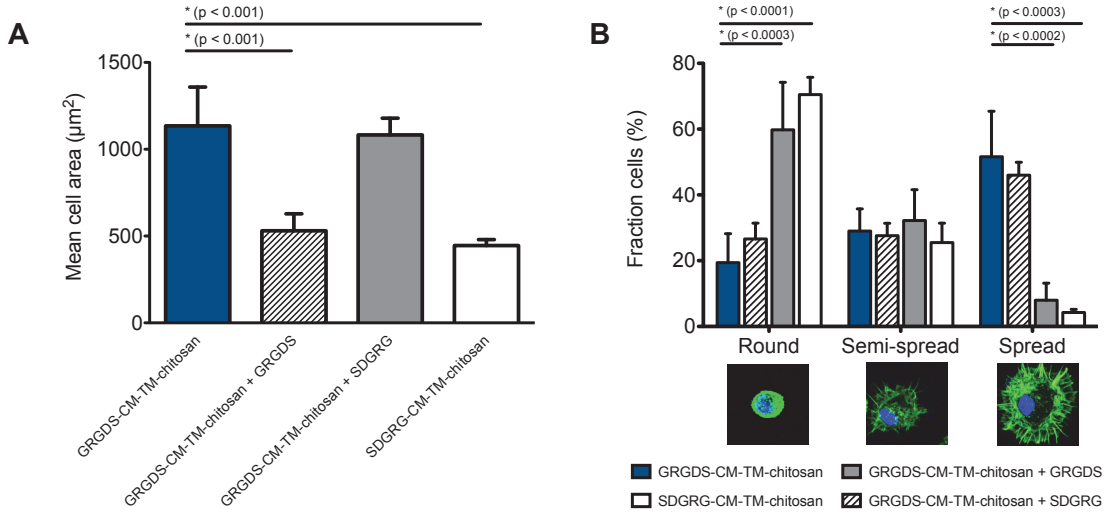


Figure 7. Cell morphology of HDF after 30 min incubation on coatings of GRGDS-CM-TM-chitosan, SDGRG-CM-TM-chitosan and on GRGDS-CM-TM-chitosan in the presence of 1 mM GRGDS- or SDGRG-peptide (competitive inhibition). (A) Mean cell area in μm^2 (n=5 assays), (B) Classification of cells based on their cell surface area, round cells (< 500 μm^2), semi-spread cells (501-1000 μm^2) and fully spread cells (>1000 μm^2). The means ± SD are corresponding to 5 assays.

The presence of the inactive sequence SDGRG did not have any significant effect on the extent of spreading. Taken together, these results suggest that in addition to increasing the level of adhesion, the GRGDS-moieties grafted onto chitosan polymer induced spreading of the cells during the early adhesion step. This is an important parameter for the cell-biomaterial interaction and also a promising sign for successive interaction.

Chitosan and its derivatives have earlier been shown to have positive effects on the wound healing process and the scar formation. Herein, we show the feasibility of adding an RGD-containing moiety to the chitosan backbone and the positive effects it brings to the early adhesion step and cell-biomaterial interaction.

Conclusions

Functionalization of a CM-TM-chitosan with an RGD peptide sequence was shown to direct the behavior of human dermal fibroblasts *in vitro* and increase interaction between the biomaterial and the surrounding tissue. GRGDS-CM-TM-chitosan was well tolerated by the cells and the RGD-moiety increased the level of adhesion of the cells. Additionally, the cells were triggered to spread during the initial adhesion and therefore increased the possibilities of a further successful cell-biomaterial interaction.

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Chapter III:

Preparation and Evaluation of Nanoparticles for Directed Tissue Engineering

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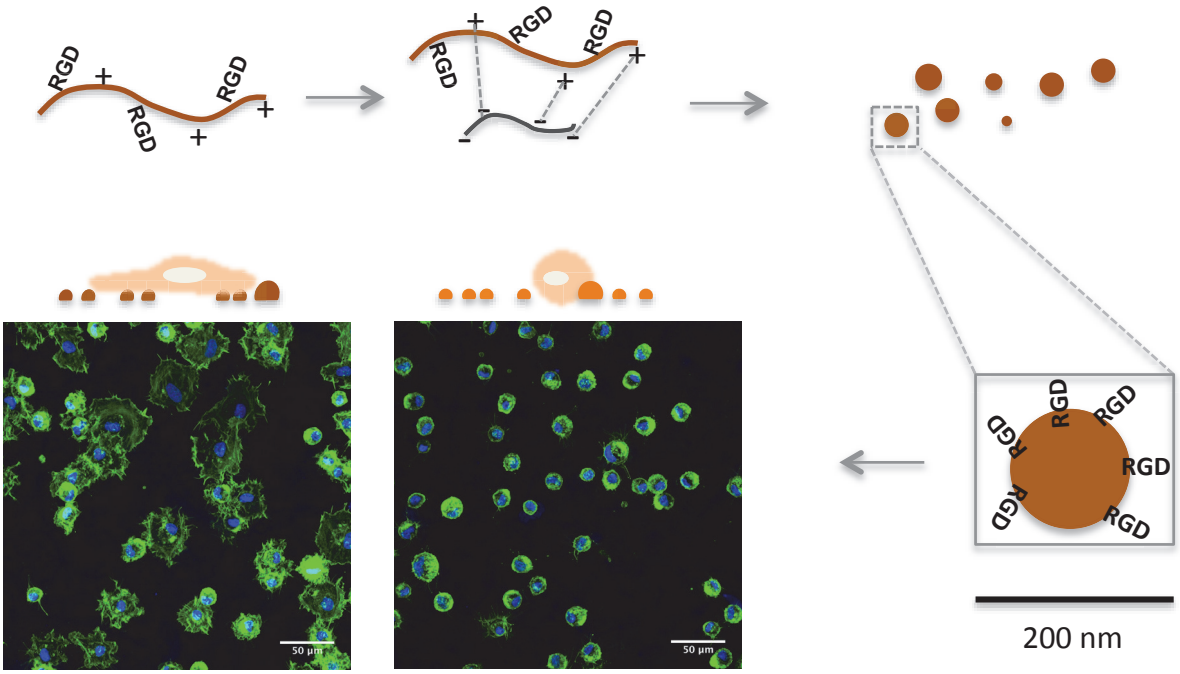
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Herein we describe the preparation of a nanoparticulate system formed from an RGD-functionalized chitosan derivative by complexation with chondroitin sulfate. These bioactive complexes were developed to promote wound healing by inducing adhesion and subsequently migration of skin cells. The particles were characterized for their size, surface charge, stability and shape. Briefly, the nanoparticles were found to be stable up to 7 days in water at a diameter of 150-200 nm and a positive charge of 20 mV. In physiological media the particles swell significantly but remain intact. Tested in an *in vitro* cell model of human dermal fibroblasts, the particles were shown to promote cell adhesion and induce spreading in human dermal fibroblasts. The mean surface area per cell was found to be increased by three-fold ($n = 3$ assays, $p < 0.01$), for the cells plated on particles exposing RGD-peptides when compared to cells on control particles. This indicates a stimulation of the cells due to the exposure of the bioactive RGD-moieties and an enhanced cell-biomaterial interaction. Using nanoparticles is a novel approach to direct cellular behavior with numerous possible applications in tissue engineering such as substrate for dermal and epithelial cells, injectable suspensions or as building blocks to form scaffolds.

Keywords: cell adhesion; chitosan derivative; human dermal fibroblast; nanoparticle; RGD peptide; wound healing

Graphical abstract



1. Introduction

In wound healing it is of major importance to protect the underlying tissue from infectious agents and extensive water loss and to help the body to rapidly restore the skin barrier. In complex wounds, such as ulcers, chronic wounds or larger burns, this normally spontaneous process might be impaired, or insufficient, and strategies to promote cell migration during the healing process might be required. An attractive strategy is to expose the cells at the wound edges to polymeric nanoparticles functionalized with adhesion peptides. The activation of the cells, inducing a change in phenotype, in combination with the particles as a substrate, would promote cell infiltration and subsequently accelerate the wound healing process.

In vivo, cell migration is induced by structural components of the extracellular matrix (ECM) and soluble factors such as growth factors and cytokines (Li et al., 2004). In 1984, Arg-Gly-Asp (RGD), a small amino acid sequence, originally identified as the cell binding sequence in fibronectin was found to induce adhesion and migration in several cell types (Pierschbacher and Ruoslahti, 1984). Since its discovery, RGD has been widely used in tissue engineering to increase cell-biomaterials interaction (Hersel et al., 2003).

A biomaterial that has gained attention over the last decades is chitosan. Chitosan is a partially deacetylated derivative of chitin, a natural polymer found in the exoskeleton of crustaceans and in the cell wall of fungi (Rinaudo, 2006). It is a polysaccharide composed of randomly distributed glucosamine and N-acetyl-glucosamine units. Being biocompatible and relative easy to modify by grafting functional groups to the polymer backbone, chitosan has been investigated as an excipient in drug delivery and in biomaterials (Alves and Mano, 2008; Baldrick, 2010). Additionally, chitosan has shown to have positive effects on wound healing (Boucard et al., 2007).

A number of different methods to prepare nanoparticles based on chitosan and its derivatives have been described, e.g., spray-drying (Learoyd et al., 2008; Mohajel et al., 2012), water-in-oil (w/o) solvent evaporation (Genta et al., 1998), w/o emulsion crosslinking (Kumbar et al., 2002; Xu et al., 2012), and ionic gelation (Fan et al., 2012; Yeh et al., 2011). Each of these methods comes with advantages and inconveniences. As an example, w/o solvent evaporation and w/o emulsion

crosslinking usually result in a high loading capacity, but require organic solvents that might be difficult to remove completely. For several of the afore-mentioned methods, chemical crosslinking is necessary and unreacted residues might have toxic effects when applied *in vitro* or *in vivo*. The most straightforward method of forming chitosan particles is ionic gelation, a one-step method under mild, aqueous conditions, i.e. no solvents or chemical crosslinkers are required. A drawback of the method is low drug loading during particle formation. Moreover, the particles formed are sensitive to changes in pH and ionic concentration, which might cause swelling and high drug release. Complexes in the nano- and micrometer-range are formed through mixing aqueous solutions of chitosan with counterions, for example smaller ions such as tripolyphosphate (TPP; (Fan et al., 2012) and sodium sulfate (Tavares et al., 2012), or larger polyionic macromolecules such as dextran sulfate (Drogoz et al., 2007), hyaluronic acid (Parajo et al., 2010) or chondroitin sulfate (Yeh et al., 2011).

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG), usually linked to proteins to form proteoglycans. CS is a linear polysaccharide composed of sulfated N-acetyl-galactosamine and glucuronic acid units. The different isoforms of CS are defined by the position of the sulfated groups. The two most common are chondroitin-4-sulfate (alternative name CS A) and chondroitin-6-sulfate (alternative name CS C) depending on the position of the sulfate group on the pyranose ring, as shown in Fig. 1. *In vivo,* sulfated GAGs are structural components abundant in the extracellular matrix (ECM), which have been proposed to play a role in wound repair, morphogenesis, and growth factor signaling (Sugahara et al., 2003). In pharmaceutical and biomedical applications, CS has been investigated in nanoparticles intended for drug delivery (Yeh et al., 2011), in wound dressings (Kirker et al., 2002), and in scaffolds in tissue engineering (Grzesiak et al., 1997; Wang et al., 2006). Due to its polyanionic nature and high charge density, CS is able to form stable polyelectrolyte complexes (PEC) with polycationic polymers either as a major component or as a crosslinker connecting the chains of the cationic polymer.

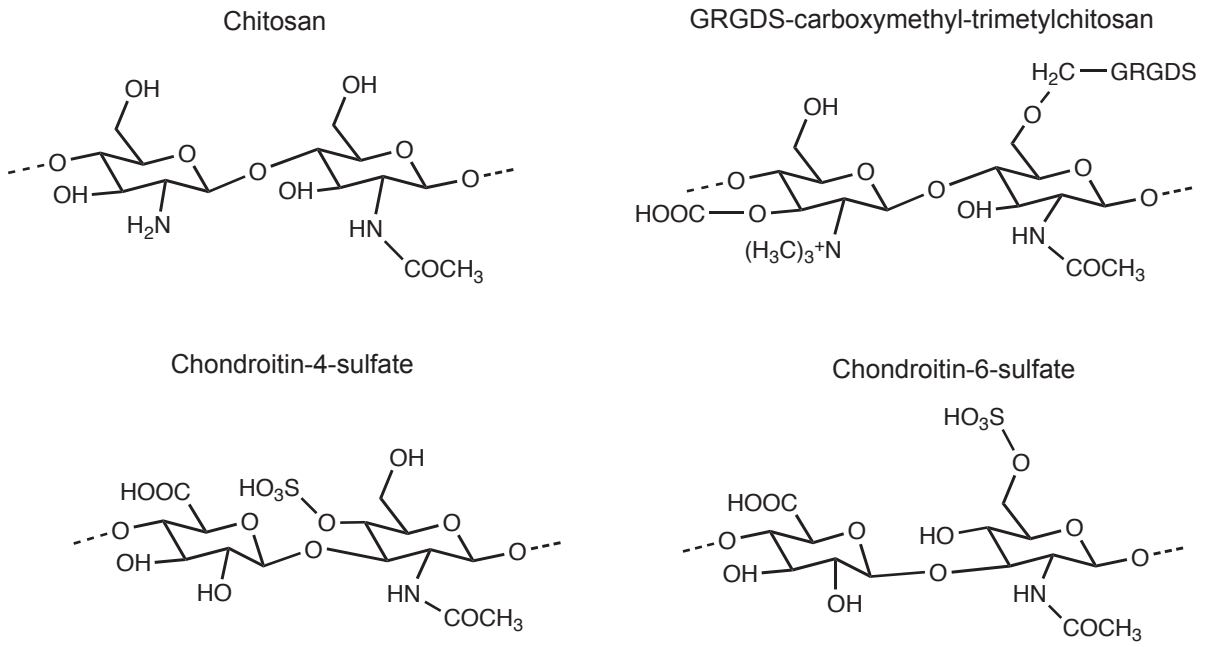


Figure 1. Chemical structures of chitosan, GRGDS-carboxymethyl-trimethyl chitosan, chondroitin-4-sulfate and chondroitin-6-sulfate.

In this present study, we propose a novel strategy to induce a change in phenotype in skin cells to promote wound healing by applying a particulate system functionalized with adhesion peptides. For this purpose, a chitosan derivative functionalized with Gly-Arg-Gly-Asp-Ser (GRGDS) peptides was synthesized. Briefly, chitosan was trimethylated to add a constant charge, carboxymethylated to add a binding site for peptides and finally GRGDS peptides were grafted to the backbone, forming GRGDS-carboxymethyl-trimethyl chitosan (GRDGS-CM-TMC) (Hansson et al., 2012). GRGDS is a pentapeptide from the cell binding sequence in fibronectin containing the bioactive RGD-sequence. As an inactive control to GRGDS, the inversed sequence SDGRG (Liu et al., 1997) was used. GRGDS-CM-TMC has previously shown to be able to induce a cell response in fibroblasts, increasing the level of adhesion and inducing spreading of the cells. Particles were formed from GRGDS-CM-TMC by complexation with chondroitin sulfate (Fig. 1). The particles were evaluated for their shape, size and charge. Additionally, their stability and swelling in physiological media was investigated. The functionality of the particles

was evaluated *in vitro* in cell adhesion assays using human dermal fibroblasts (HDF). The HDF were chosen as a cell model due to their documented interaction with RGD-peptides through the integrin $\alpha V\beta 3$, mediating signals promoting attachment and spreading of the cells (Massia and Hubbell, 1991). Herein, we investigate the feasibility to formulate the polymer into nanoparticles for further pharmaceutical and clinical applications.

2. Materials and methods

2.1 Polymer synthesis

Carboxymethyl-trimethyl chitosan (CM-TMC), GRGDS- and SDGRG-CM-TMC were synthesized from chitosan (ChitoClear Cg10, 7-15 mPa·s; Primex, Siglufjordur, Iceland) in two- and three-step synthesis, respectively (Hansson et al., 2012). Briefly, chitosan was trimethylated through nucleophilic substitution by methyl iodide to form N-trimetylchitosan (TMC). Subsequently carboxymethyl groups were added by chloroacetic acid to form CM-TMC. In a final step, GRGDS and SDGRG moieties (both from Bachem, Bubendorf, Switzerland) were grafted to CM-TMC via the carboxylic groups using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich, Buchs, Switzerland) and N-hydroxysuccinimide (NHS; Sigma-Aldrich). The degree of substitution of trimethylation and carboxymethylation was determined through H^1-nuclear magnetic resonance (H^1-NMR) using a Varian Gemini 300 MHz spectrometer (Agilent Technologies, Santa Clara, USA) and the density of GRGDS- respective SDGRG-moieties through amino acid analysis (AAA; Biochrom 30, Biochrom Ltd., Cambridge, UK).

2.2 Polyelectrolyte complex formation

CS (C-4-S, min. 60 %, and C-6-S) was obtained from Sigma-Aldrich GmbH (Buchs, Switzerland). Solutions of the polymers were prepared by solubilizing the CM-TMC (5 mg/ml), GRGDS-CM-TMC (0.5 mg/ml) and CS (1.5 resp. 0.15 mg/ml) in Milli-Q water. The solutions were filtered through 0.22 µm centrifugal filters (Carl Roth, Lauterbourg, France). One ml of the CS solution was successively added to 1 ml of the solutions containing the chitosan derivative by a one-shot addition at ambient temperature (Schatz et al., 2004). The dispersion was vortexed before the particles

were separated from the supernatant by centrifugation (10 min, 20817 x g; Eppendorf 5810 R, Vaudaux-Eppendorf, Basel, Switzerland). The supernatant was removed and the pellet was resuspended in Milli-Q water. The yield of particles was determined as 5 % after drying the nanoparticles at 50 ºC until constant weight.

2.2 Storage and stability assays

For the evaluation of stability of the formed nanocomplexes in water, particle size was determined immediately after centrifugation, after 24 hours and 7 days. Particles were lyophilized in 1 % trehalose (Fluka) and resuspended in Milli-Q water before being characterized. Moreover, the stability of complexes in physiological media was assessed by dispersing centrifuged nanoparticles in NaCl 0.9 %, PBS pH 7.4 (PAN Biotech GmbH, Aidenbach, Germany), Dulbecco's Modified Eagle Medium (DMEM; PAN Biotech GmbH) and DMEM complemented with 10 % fetal calf serum (FCS; PAN Biotech GmbH). The particle size was measured after 30 min and 24 h.

2.3 Characterization of nanoparticles

2.3.1 Photon correlation spectroscopy

The nanocomplexes were characterized by photon correlation spectroscopy (PCS) using a Malvern ZetaSizer Nano SZ (Instrumat SA, Renens, Switzerland) equipped with a 10 mW He/Ne laser beam operating at λ = 633 nm. Z-average, polydispersity index (PDI) and Zeta-potential (ζ) were determined. For a monodisperse distribution, the polydispersity index (PDI) should be <0.05, however, values up to 0.5 can be used for comparison purposes (Drogoz et al., 2007).

2.3.2 Scanning electron microscopy

The morphology of dried nanoparticles was imaged with scanning electron microscopy (SEM; JEOL JSM-7001FA, Tokyo, Japan) at an acceleration voltage of 5-15 kV. The samples were sputter coated with 10 nm gold to reduce charging and damage of the sample by the beam.

2.3.3 Differential interference contrast microscopy

Hydrated, swollen particles were imaged by differential interference contrast (DIC) microscopy using a Zeiss Axio Imager Z1m microscope (Carl Zeiss AG, Feldbach,

Switzerland) equipped with an Axiocam MRm camera (Carl Zeiss AG). An x100 oil objective was used for imaging.

2.4 Cell culture

Human dermal fibroblasts (HDF) were a kind donation by Dr. Lee-Ann Laurent-Appelgate (CHUV, Lausanne, Switzerland). All cell culture media and additives were bought from PAN Biotech GmbH (Aidenbach, Germany). The HDF were cultured in DMEM containing 2 mM glutamine, 10% FCS and 1% penicillin/streptomycin. The cells were split at sub-confluency with trypsin/EDTA diluted in PBS. The HDF were used between passages # 6 and 10.

2.5 Cytotoxicity

Potential cytotoxicity of the nanoparticles on HDF was assessed using an XTT-cell proliferation kit 2 (Roche, Basel, Switzerland). Cells were seeded in a 96-well plate (Costar, Corning, Amsterdam, The Netherlands) at an initial concentration of 15 x 10^3 cells/well. After 24 hours the medium was changed to fresh medium containing nanoparticles at concentrations of 1, 0.5, 0.25 and 0.125 mg/ml. The cells were cultured in the presence of the nanoparticles for 24 hours before removing the medium and adding XTT-reagent. The absorbance was measured at a wavelength of 490 nm after 6 h using a Tecan plate reader (Tecan Group Ltd., Männedorf, Switzerland) and the cell viability was expressed in percentage relative to a control group of HDF cultured under the same conditions in the absence of nanoparticles.

2.6 Adhesion assays

Drops of dilute dispersions of nanoparticles (0.1, 0.05 and 0.025 mg/ml) formed from GRGDS- or SDGRG-CM-TMC with CS were placed on cover glasses and let dry over night. The wells were subsequently saturated with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1h at ambient temperature. The HDF were detached and rinsed in serum-free DMEM before being seeded at a density of 10^5 cells/well. After 30 min of incubation (37˚C, 5% CO_2), the non-adhered cells were removed by washing with PBS and the attached cells were fixed for 1 hour with a solution of 1% glutaraldehyde (Sigma-Aldrich) in PBS. To evaluate the level of adhesion, the cells were stained for 1 hour with a solution of 0.1 % crystal violet. To assess the cell number and mean cell surface area, microscopic images were analyzed with ImageJ software (available from National Institutes of Health, Bethesda, MD, USA).

2.7 Immunofluorescence staining

For morphological analysis, the attached cells were fixed in pre-warmed PBS containing 4% paraformaldehyde (Alfa Aesar, Ward Hill, USA) and permeabilized with 0.1% Triton X-100 in PBS for 3 min after cell adhesion assays. Samples were then pre-incubated with 1% BSA in PBS for 20 min, before being incubated with 6.6 µM phalloidin conjugated to Alexa 488 (Lonza, Basel, Switzerland) in 1% BSA in PBS for 20 min. After being washed with PBS, the fixed and stained cells were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd., Peterborough, UK) before being attached to a microscopic slide. The cell morphology was analyzed with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss AG, Feldbach, Switzerland) using a x20 objective. The images were treated and analyzed with Image J software (NIH).

2.8 Statistical significance

Statistics for nanoparticles were assessed through analysis of variance (ANOVA) 1-way and 2-way assays performed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, USA), followed up by Tukey's Multiple Comparison Test (all pairs of columns compared), respectively Bonferronis Multiple Comparison-post test. The significance level was chosen to $p < 0.05$. The statistical significance for all *in vitro* assays was assessed by student's t-test (for two samples, assuming equal variance) comparing data at a significance level of $p < 0.05$. The results were expressed as mean ± standard deviation.

3. Results and discussion

3.1 Nanoparticle preparation and characterization

The aim of the present work was to prepare nanocomplexes functionalized with RGD-peptides and to evaluate their ability to induce a change in phenotype of human dermal fibroblasts. A cationic, water-soluble chitosan derivative functionalized with GRGDS-peptide, GRGDS-CM-TMC, was synthesized with the aim of forming PECs. The synthesized polymers are cationic chitosan derivatives with constant charge due to the trimethyl-groups. Through H^1-NMR, CM-TMC and its functionalized derivatives, were determined to have a N-trimethylation of 0.4 and

with a total O-carboxymethylation of approximately 0.3. The AAA showed a density of 69 nmol/mg of GRGDS respectively 59 nmol/mg for SDGRG grafted to the CM-TMC backbone.

Nanocomplexes were prepared through complex coacervation between the catonic polymers CM-TMC, GRGDS- and SDGRG-CM-TMC, with the polyanionic CS. The particles were characterized for their size, PDI and charge by PCS, showed in Table 1. The mean diameter of the particles is expressed in Z-average (nm) and the charge as Zeta-potential (mV). SDGRG-CM-TMC particles exhibited the same properties as those prepared with GRGDS-CM-TMC and is therefore not shown. Firstly, all particle types showed similar properties and remained of constant size during all types of handling (1-way ANOVA, $p > 0.05$) under all conditions tested. The particles were of a size range of 150-200 nm before and after centrifugation. The particles were shown to be stable when suspended in water and their size remained in this range after storage in suspension for both 24 h and 1 week at room temperature. Additionally, lyophilized nanocomplexes resuspended in water were measured and showed a constant size. No significant difference in size was observed between the particles formed out of CM-TMC and GRGDS-CM-TMC when comparing between treatment pairs (2-way ANOVA, $p > 0.05$). However, an overall significant size decrease for particles of GRGDS-CM-TMC, with respect to the CM-TMC particles, is seen when considering all treatment groups (2-way ANOVA, $p < 0.01$). For particles, remaining stable under the conditions of separation techniques such as centrifugation and drying by lyophilization in view of long time storage are important aspects for further pharmaceutical development. The surface charge of the particles was positive, ranging between +20 and +30 mV. A slight decrease was seen after centrifugation of the particles and is probably due to removal of loosely bound CM-TMC and GRGDS-CM-TMC chains from the particle surface.

	CM-TMC			GRGDS-CM-TMC		
	Size (nm)	PDI	ζ (mV)	Size (nm)	PDI	ζ (mV)
Before centrifugation	191± 32	0.2-0.3	30 ± 4	162 ± 21	0.1-0.3	29 ± 7
After centrifugation	188 ± 43	0.1-0.2	25 ± 3	172 ± 4	0.2-0.3	20 ± 1
24 h in H_2O	173 ± 16	0.1-0.2	21 ± 3	156 ± 6	0.2	17 ± 6
7 days in H_2O	177 ± 33	0.1-0.2	18 ± 3	156 ± 3	0.2	21 ± 4
Lyophilised particles	179 ± 6	0.1-0.2	31 ± 8	158 ± 13	0.1-0.3	32 ± 5

Table 1. Properties of nanocomplexes formed of CM-TMC and GRGDS-CM-TMC through complexation with chondroitin sulfate. The size in nm and Z-potential (ζ) in mV of the nanocomplexes were determined before and after centrifugation, after storage in water and after lyophilisation (minimum n = 5 batches for CM-TMC and n = 3 batches for GRGDS-CM-TMC).

Preparations of nanocomplexes of two oppositely charged polymers, formed by complexation may be done either in excess of the one or the other. Herein, cationic particles composed of mainly GRGDS-CM-TMC with CS added as a crosslinker, were prepared. GRGDS-CM-TMC was used as a base to increase the density of GRGDS-moieties on the particle surface. The molar ratio of the charged groups of 2.7 (n^+/n^-) was found to be optimal for the preparation. This ratio was obtained by calculations based on the number of $N^+(CH_3)_3$ groups in CM-TMC and SO_{3^-} groups in CS, neglecting the weaker ionic interactions between $COO^-$and $N^+(CH_3)_{3.}$. Reports of complex formation between chitosan and sulfated polymers have shown that flocculation occurs using charge ratios close to unity (Boddohi et al., 2009; Drogoz et al., 2007). A particle yield of 5% (mass) was obtained at the present ratio, probably limited by the CS mass used in the preparation (30% of the counter polymer). However, at this ratio the particles kept a low PDI, showed no flocculation tendency and were re-suspendable after centrifugation and lyophilization.

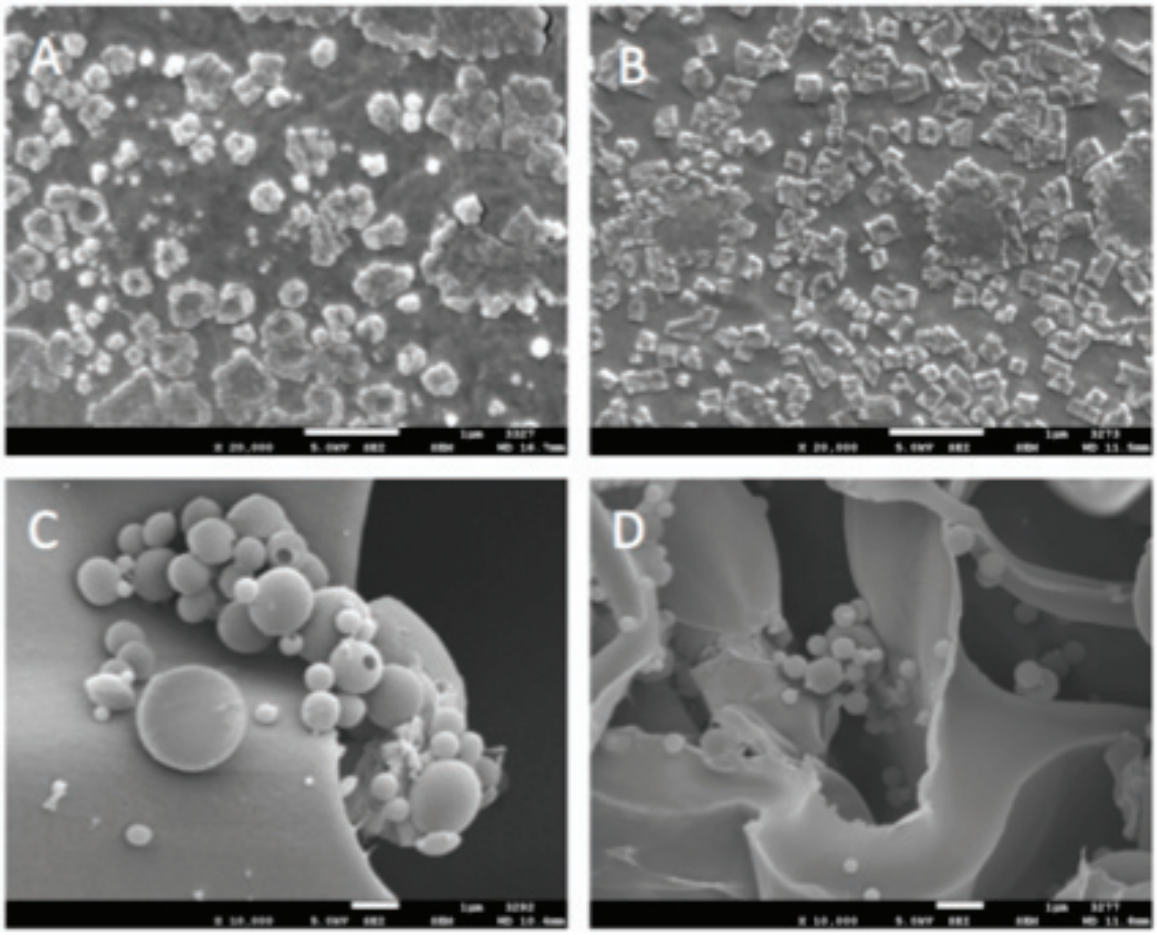


Figure 2. Scanning electron microscopy images of CM-TMC/CS (A and C) and GRGDS-CM-TMC/CS (B and D) nanocomplexes. A-B shows air-dried particles and C-D lyophilised particles. The scale bars represent 1 μm.

To further investigate the morphology of the nanocomplexes, scanning electron micrographs of the complexes in dry form were taken, as shown in Fig. 2. Two different drying methods were applied, air-drying at room temperature or an initial freezing of the suspension and subsequent lyophilization. The air-dried particles were observed as crystal-like structures for particles prepared using CM-TMC or GRGDS-CM-TMC. The size of the particles corresponded to the values obtained by PCS. Particles were of spherical shape after lyophilization. Hydrogel plane structures seen in the background of the lyophilized samples SEM are only seen in the absence of cryoprotectant, which is avoided here to allow particle imaging. There was no difference between the particles dried in the same manner formed from CM-

TMC or GRGDS-CM-TMC. However, the particles formed by air-drying were of cubical symmetry with sharper angles, whereas the lyophilized samples were spherical, indicating a re-arrangement during the air-drying process. Chitosan is a semi-crystalline polymer (Rinaudo, 2006), whereas CS is an amorphous biomolecule (Yao and Wu, 2010). The shape of the dry particles formed after air-drying indicates that the derivatives, CM-TMC and GRGDS-CM-TMC, conserve the semi-crystalline form. Additionally, observations of increased crystallinity of mixtures of chitosan and CS compared to chitosan alone have been reported (Piai et al., 2009; Yao and Wu, 2010). In their hydrated state, however, it is probable that the particles take on a more spherical form as seen in the lyophilized samples.

3.2 Nanoparticles in physiological media

The main objective of the assays studying the mean diameter of the particles after incubation in physiological media was to verify the stability of the particles. Increasing the ionic concentration of the external phase might induce swelling, aggregation or dissociation. When added to physiological media (0.9 % NaCl, PBS pH 7.4, DMEM or DMEM complemented with 10 % FCS), a significant increase in particle size was observed for both types of particles compared to particles in H_2O (Table 2; 1-way ANOVA, $p < 0.01$). The swelling-behaviors of the two types of particles were similar (2-way ANOVA, $p > 0.05$). The particle sizes were 5-fold and 8-fold larger when suspended in 0.9 % NaCl and PBS, respectively, after 30 min. After 24 h incubation the particles showed 10- respectively 20-fold larger diameters compared to the particles stored in water for 24 h, representing a significant increase compared to after 30 min (2-way ANOVA, $p < 0.05$). Also the particles in DMEM showed an increase in size over time, however the increase is not significant (1-way ANOVA, $p > 0.05$). For particles incubated in NaCl 0.9 % a positive surface charge of approx. 20 mV was measured, whereas the particles in PBS showed a negative charge of -20 mV. In cell culture medium, the particles showed a similar swelling profile as in NaCl 0.9 % with a negative surface charge of approximately -20 mV. The size of particles prepared with GRGDS-CM-TMC in DMEM/10 % FCS is not shown (n.s.) due to a resulting bimodal distribution curve. Aggregates of a size of a

few nanometers, attributed to the serum, were observed in addition to a peak in the expected size range of 1000-2000 nm and decreased the overall Z-average value.

	CM-TMC			GRGDS-CM-TMC		
Medium	**Size (nm)**	**PDI**	**ζ (mV)**	**Size (nm)**	**PDI**	**ζ (mV)**
H_2O	188±43	0.1-0.2	25 ± 3	172±4	0.2-0.3	20 ± 1
NaCl 0.9 %						
30 min	862±225	0.3-0.5	20 ± 1	869 ± 43	0.3-0.4	17 ± 0
24 h	1707 ± 556	0.4-0.8	27 ± 2	1830 ± 360	0.4-0.5	21 ± 1
PBS pH 7.4						
30 min	1419 ± 251	0.3-0.5	-25 ± 3	1387±71	0.2-0.3	-17 ± 1
24 h	2520 ± 678	0.2-0.3	-27 ± 1	3299 ± 503	0.1-0.4	-17 ± 5
DMEM						
30 min	1422 ± 81	0.2-0.3	-22 ± 1	1065 ± 347	0.5	-16 ± 2
24 h	1954 ± 282	0.3-0.4	-18 ± 1	1727 ± 380	0.3-0.6	-14 ± 1
DMEM+FCS 10%						
30 min	1517 ± 163	0.4	-22 ± 1	n.s.		n.s.
24 h	1751 ± 998	0.3-0.5	-18 ± 1	n.s.		n.s.

Table 2. Stability of nanoparticles formed from CM-TMC and GRGDS-CM-TMC through complexation with chondroitin sulfate. Nanocomplexes were incubated in PBS pH 7.4, NaCl 0.9%, DMEM and DMEM complemented with 10 % serum and the size and Z-potential (ζ) was measured at 30 min and 24 h.

The increase in size might be attributed to the swelling of the particles and/or to aggregation. Due to the relatively low PDI-values of the dispersions (PDI < 0.5 for most samples), and the absence of flocculation in the samples, it was suggested that the increase in size was due to swelling of the polymeric network. The presence of ions in the medium weakens the electrostatic interactions between the anionic and cationic groups and leads to a looser network allowing more water to enter. The swelling of chitosan NP is highly dependent on the pH and the ionic concentration of

the external phase (Berger et al., 2004a; Berger et al., 2004b). Additionally, Dautzenberg (1997) has demonstrated that a very high degree of swelling occurs at ratios (n^+/n^-) ranging from 2 to 3.3 in the presence of ions during the formation of polyelectrolyte complexes (PECs). Given the fact that the ratio estimated for our complexes, 2.7 (n^+/n), is in the reported range, this supports the hypothesis of an increase in size due to swelling of the complexes. On the other hand, the critical coagulation concentration for PECs formed of chitosan and dextran sulfate has been determined to be approximately 0.1 M NaCl (Schatz et al., 2004). The concentration of 0.9 % NaCl corresponds to 0.15 M, thus theoretically the increase in size of the particles could be at least partially due to aggregation despite the absence of flocculation or precipitation.

To further investigate the origin of the increase in size, the morphology of the swollen particles was analyzed by differential interference contrast (DIC) microscopy. Particles were incubated in physiological media (NaCl 0.9% and PBS) and cell culture medium (DMEM with and without FCS) for over 1 h before being observed. As seen in Fig. 3, spherical particles with a diameter in the size range of 1000-3000 nm were observed for all four different media confirming the hypothesis of extensive swelling of the particles bringing them from the nano- into the micrometer-range. Particles suspended in PBS tended to form aggregates, however, the individual particles remained visible in the aggregates. The phenomenon of particle aggregation in PBS is not seen with PSC and it’s therefore plausible that the highly swollen particles are sensitive to higher concentrations and handling necessary for microscopy imaging.

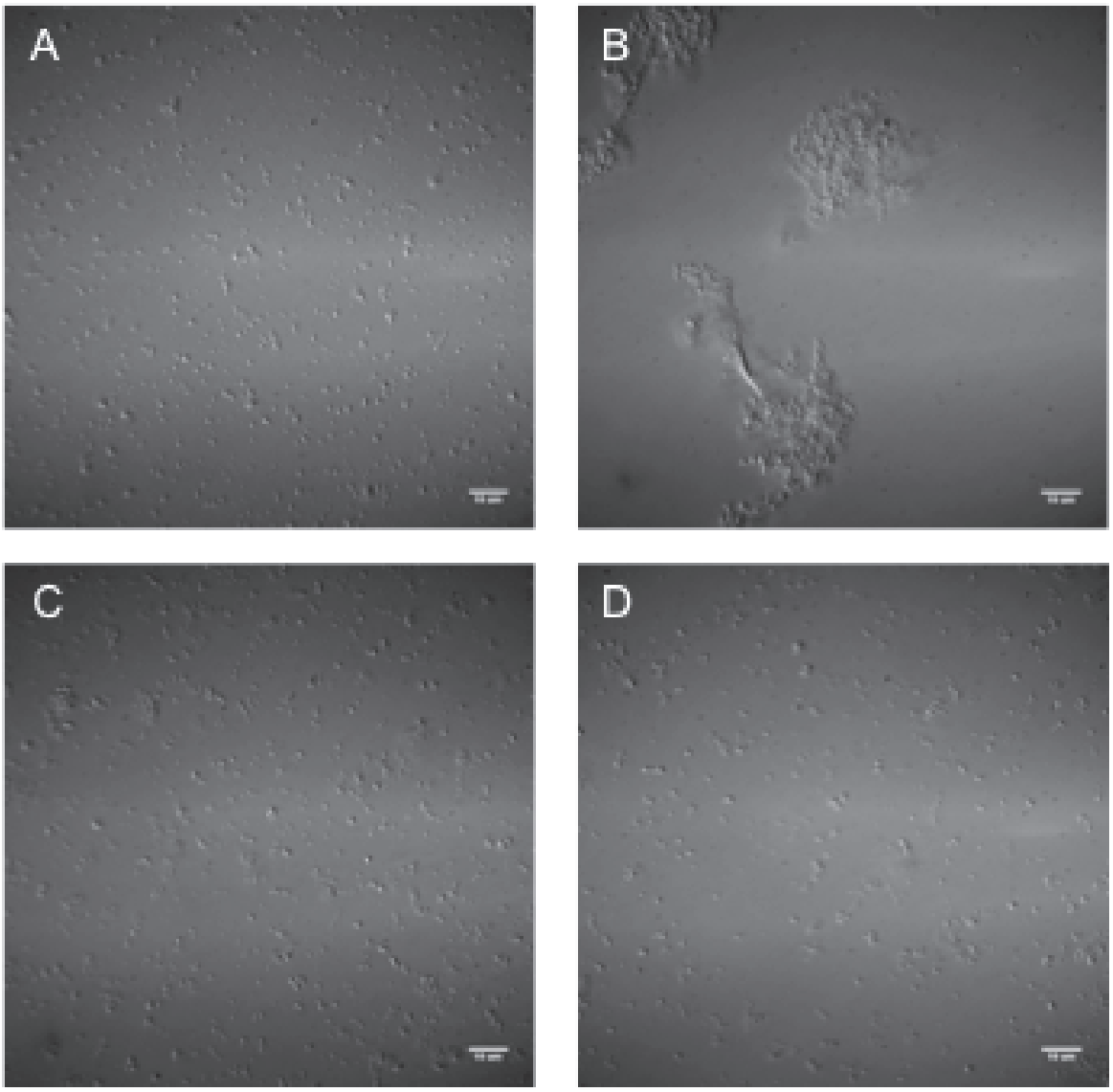


Figure 3. DIC images of swollen CM-TMC particles incubated > 1 h in physiological media. (A) NaCl 0.9 %, (B) PBS pH 7.6, (C) DMEM and (D) DMEM complemented with 10 % serum. The scale bars represent 10 µm.

3.3 In vitro characterization

3.3.1 Cell viability

As a first step of investigating the nanoparticles for their activity *in vitro*, cytotoxicity assays were performed to exclude toxicity of the nanoparticles towards HDF. The viability of the cells was determined after 24 h in culture in the presence of CM-TMC nanoparticles, as shown in Fig. 4. The cell viability decreased slightly with increasing

concentrations of nanoparticles, from >90% at 0.1 mg/ml to approximately 80% at 1 mg/ml. The cell viability for the concentrations used in this study was > 90 % and the particles were therefore determined to be safe to be used in further *in vitro* experiments. For technical reasons, high concentrations of particles of GRGDS- and SDGRG-CM-TMC, respectively, could not be obtained. However, the polymers in their soluble form have shown similar cell viability profiles as the CM-TMC particles in previous tests and similar or higher cell viability profiles could be expected from the functionalized particles.

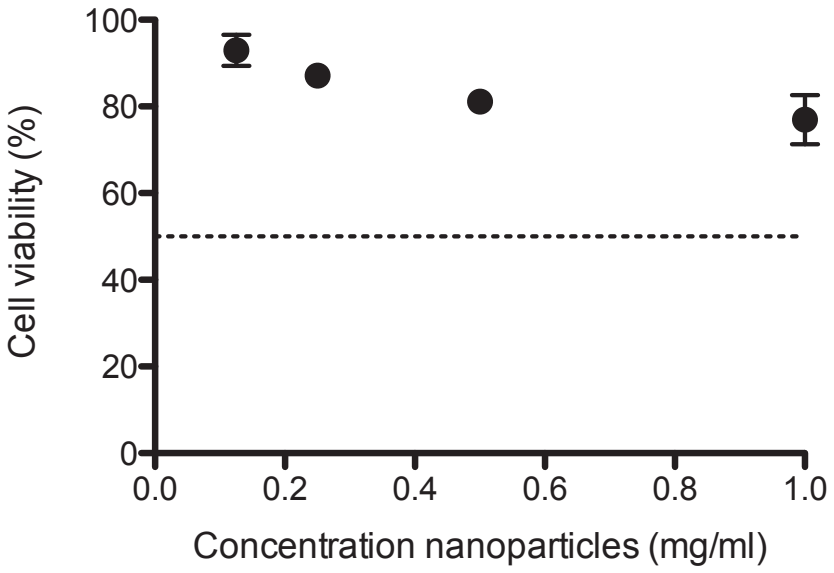


Figure 4. Cell viability of HDFs after 24 h incubation with CM-TMC particles at concentrations 0.125, 0.25, 0.5 and 1 mg/ml.

3.3.2 Cell adhesion

In further *in vitro* assays the bioactivity of particles formed from GRGDS- and SDGRG-CM-TMC, respectively, were evaluated for their ability to induce cell adhesion in human HDF. Briefly, dilute suspensions of particles were air-dried on coverslips and the ability of the cells to adhere was tested through adhesion assays. The level of adhesion was determined by analyzing microscopic images of cells dyed

with crystal violet and the morphology of the cells was investigated through immunofluorescence staining and confocal microscopy. The first observation indicated that the cells were found to attach only to the regions covered by both GRGDS- and SDGRG-CM-TMC particles. Furthermore, no difference in cell number on the different coatings was observed. Comparing the cell number on surfaces coated with GRGDS-conjugated particles with SDGRD-particles, ratios of 1.0 ± 0.2, 1.0 ± 0.2 and 1.0 ± 0.5 were found for concentrations 0.1, 0.05 and 0.025 mg/ml, respectively (cells on GRGDS-particle coatings/cells on control coatings; 250-450 cells/condition and assay; n = 5 assays). Despite a significant swelling, the matrix stays sufficiently stable to sustain cell adhesion, indicating adequate mechanical properties for tissue engineering.

3.3.3 Cell spreading

Although no difference in cell number was seen, a distinct difference in cell phenotype was observed on the coatings with GRGDS when compared to the control coatings. By staining the actin-fibers in the cytoskeleton, the morphology of the cells was observed through confocal microscopy, as seen in Fig. 5A and 5B. After 30 min of incubation on coatings of GRGDS-CM-TMC nanoparticles, the majority of cells were fully spread. For the cells plated on the particles containing the control polymer SDGRG-CM-TMC, the cells remained spherical and little or no spreading was observed. HDF were previously confirmed to attach and spread when plated directly onto GRGDS-CM-TMC polymers. Here it was demonstrated that the cells responded in a similar manner to the nanoparticles with a high degree of spreading.

To quantify this difference, the mean surface area per cell was measured using the imaging software Image J. A three-fold larger cell surface area was found after 30 min for the cells plated on coatings of GRGDS-CM-TMC particles compared to the control coatings. As seen in Fig. 5C, the mean cell surface area for the cells on coatings including GRGDS-peptides were in the range of 700-800 μm^2, whereas the cells on particles with SDGRG-particles had a mean surface area of 250 μm^2 (n = 3 assays, $p < 0.01$). The size of the spread cells showed a broad distribution at a variation coefficient of 45-75 %. The mean cell size was independent of the coating

concentration and no significant variations were observed between the different concentrations (p = 0.4). These results confirmed that the GRGDS-moieties in GRGDS-CM-TMC are exposed and preserved their activity after nanocomplex formation.

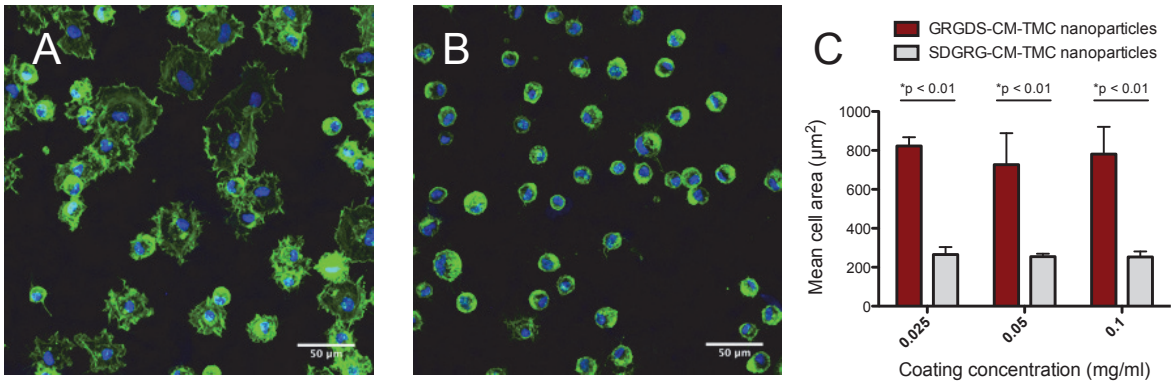


Figure 5. Confocal images of HDFs and quantification of spreading after adhesion assays. The morphologies of HDFs on coatings of 0.1 mg/ml (A) GRGDS- and (B) SDGRG-CM-TMC nanoparticles, were visualized by immunofluorescence staining. The actin-fibers are shown in green and the nuclei in blue. The images are taken at x20 magnification and the scale bar represent 50 µm. (C) The mean cell surface area of HDFs plated on coatings of GRGDS- and SDGRG-CM-TMC particles, respectively. Three concentrations were tested, 0.025, 0.05 and 0.1 mg/ml. The value represent the mean of 5 assays and the error bars indicate the standard deviation.

The aim of this work was to develop bioactive nanoparticles to be used to promote adhesion and migration in skin cells. Due to the known interaction between HDF and RGD-sequences, HDF were chosen as a suitable cell model to prove that the activity of GRGDS-CM-TMC was preserved after complex formation with CS. In wound healing, HDF infiltrate the granulation tissue and migrate in a three-dimensional manner, hence in dermal tissue engineering, an appropriate scaffold is needed. Recently, microengineered particle-based scaffolds have gained interest as they form appropriate structures for cell support (Khademhosseini and Langer, 2007;

Miranda et al., 2011). Using nanocomplexes in tissue engineering is a novel approach with the advantages of deformability, scaffold formation, defined pore size and the ability to follow the surface structure of the wound bed and fill voids in the damaged tissue. Herein we propose a particular system to direct tissue development for dermal cells with the possibility of being further developed to suit other areas of tissue engineering.

4. Conclusions

The aim of the present study was to develop particles functionalized with RGD-peptides to be used to direct cell behavior. The nanoparticles formed through complex coacervation between an RGD-functionalized chitosan derivative and CS were shown to be stable and to support cell adhesion and spreading. Overall, particles formed by complexation between GRGDS-CM-TMC and CS showed promising properties for directing tissue restoration and should be further investigated *in vitro* with other skin cells before *in vivo* assessments.

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Abbrevations: Arg-Gly-Asp (RGD); carboxymethyl-trimethyl chitosan (CM-TMC); chondroitin sulfate (CS); differential interference contrast (DIC); fetal calf serum (FCS); Gly-Arg-Gly-Asp-Ser (GRGDS); human dermal fibroblast (HDF); photon correlation spectroscopy (PCS); polyelectrolytic complexes (PEC); Ser-Asp-Gly-Arg-Gly (SDGRG); tripolyphosphate (TPP);

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Chapter IV:

In vitro Evaluation of the Interaction Between HaCaT-Cells and GRGDS Functionalized Carboxymethyl-Trimethyl Chitosan

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In wound healing, re-epithelialization is one of the essential steps to complete tissue restoration and to protect the underlying tissue against extensive water-loss and infectious agents. Herein, we investigated the possibility of using a GRGDS-functionalized chitosan derivative, carboxymethyl-trimethylchitosan, to induce adhesion in keratinocytes with the aim of promoting migration and subsequently favor re-epithelialization. Immortalized keratinocytes (HaCaT) were used to evaluate the interaction between the cells and polymers. The level of adhesion and the spreading of the cells were assessed when exposed with the functionalized polymers and compared with an inactive control polymer. No difference in level of adhesion or morphology of the cells exposed to GRGDS-polymers could be distinguished. Explanations such as insufficient peptide concentrations or weak interactions between RGD and the RGD dependent integrins have been suggested and further *in vitro* investigations are needed.

Keywords: Cell adhesion; HaCaT; RGD peptides; chitosan derivative

1. Introduction

Keratinocytes are the main cell type forming the epidermis, the barrier protecting the body from external mechanical stress, infectious agents and water-loss. The keratinocytes form a stratified epithelium of cells tightly connected to the neighboring cells or the extracellular matrix. During the re-epithelialisation of a wound, the keratinocytes at the wound edges and in the hair follicles are released from these connections and change their phenotype to the migratory state (Ortonne et al., 1981). This change in phenotype is due to an exposure of new extracellular matrix (ECM) components and the production of growth factors and cytokines by the immune cells recruited to the wound. *In vivo*, cell migration is a well-organized process regulated by insoluble factors of the ECM, such as fibronectin, laminin and collagen, as well as soluble growth factors (GF) such as epidermal growth factor (EGF), keratinocyte growth factor (KGF) and insulin-like growth factor-1 (IGF-1), to mention a few (Li et al., 2004a). The insoluble ECM-structures have been shown to induce migration, whereas GFs increase and give the directionality of the migration (Li et al., 2004b). During wound healing, fibronectin is deposited on the wound surface (Grinnell et al., 1981), forming the granulation tissue. The keratinocytes thus migrate on a matrix rich in fibrin and fibronectin (Clark et al., 1982). Lacking the $\alpha V\beta 3$ integrin, keratinocytes are unable to interact with fibrin. This mechanism has been suggested to be responsible for the dissection of the fibrin clot and lining the viable tissue of the keratinocytes during the re-epithelialization step (Kubo et al., 2001).

The main cell receptors responsible for migration are members of the integrin family (Ridley et al., 2003). Integrins are transmembrane cell surface receptors composed of two subunits, α and β (Hynes, 2002). The composition of different α- and β-units confers the specificity of the integrin. Twenty four different integrins were identified in humans, which are able to transmit signals bidirectionnally across the cell membrane. Intracellularly, the receptor is connected to the cytoskeleton through over 50 different associated proteins (Zamir and Geiger, 2001). Binding with a ligand to the integrin receptor, triggers a cascade of downstream signals, resulting in the rearrangement of the cytoskeleton and inducing spreading and migration.

Fibronectin is an ECM-protein, known to induce cell migration and adhesion in cells. It is a mosaic protein composed of three types of modules, type I, II and III. The

interactions between cells, fibronectin and integrins are mainly mediated through a small amino acid sequence located in the 10th type III module, Arg-Gly-Asp (RGD). In addition to the RGD-sequence, a pentapeptide, Pro-His-Ser-Arg-Asn (PHSRN) in the 9th type III module acts as a synergy site, stabilizing the interaction between fibronectin and certain integrins, e.g., α5β1 (Aota et al., 1994; Obara et al., 1988). Ever since the discovery of the RGD and the fact that the short sequence is able to induce a response similar to the whole protein (Pierschbacher and Ruoslahti, 1984), several different roles have been defined for RGD. Keratinocytes express a number of different integrins recognizing RGD as their ligand (Margadant et al., 2010), see Table 1.

Integrin		Ligand	Expression in epidermis
β1			
	α2	Collagens	Constitutive, up-regulated during wound healing
	α3	Laminins	Constitutive, up-regulated during wound healing
	α5	FN (or RGD in FN)	Induced during wound healing
	α6	Laminins	Uncertain
	α8	RGD	In developing HF and the arrector pili muscle
	α9	RGD, mainly FN and TN	Weak constitutive, up-regulated during wound hesling
αV			
	β1	RGD	Uncertain
	β5	RGD, mainly VN	Weak constitutive, up-regulated during wound healing
	β6	RGD, mainly FN, TN and LAP	In stem cells in the HF, induced during wound healing
	β8	RGD, mainly VN, TN and LAP	Weak, suprabasal
α6	β4	Laminins, mainly LN-332	Constitutive

Table 1. Expression of integrins in the epidermis and their ligands. Abbrevations: FN, fibronectin; HF, hair follicle; TN, tenascin; VN, vitronectin; LAP, latency-associated protein. Table modified from Margadant et al. (2010).

Under normal conditions, wound healing is a spontaneous, temporally and spatially organized process (Singer and Clark, 1999). However, in complex wounds or larger wounds, strategies are needed to promote the healing process. Using nanoparticles formed of an RGD-functionalized polymer is a novel strategy to provide a scaffold or a substrate for the keratinocytes to migrate on during the re-epithelialization step. The realization of this work was divided into 3 steps; (i) development of RGD-functionalized chitosan derivative and *in vitro* evaluation with human dermal fibroblasts (HDF), (ii) formation of nanoparticles and (iii) *in vitro* evaluation of the keratinocyte interaction with the RGD-polymer. HDF was chosen as a cell model to confirm the RGD-activity by a cell line known to interact with the RGD-sequence through the integrin $\alpha V\beta 3$. In the previous steps, the developed RGD-functionalized polymer was shown to promote adhesion of HDF through specific RGD interaction. In the present work, the *in vitro* evaluation of the RGD-functionalized polymer, GRGDS-carboxymethyl-trimethyl chitosan (GRGDS-CM-TMC), with epidermal keratinocytes is presented.

To interact with integrins and induce adhesion and spreading, RGD needs to be given structure, for example by a polymeric backbone. Chitosan is a natural polysaccharide obtained from the deacetylation of chitin and composed of a mixture of glucosamine and N-acetyl-glucosamine units (Illum, 1998). Generally, the definition of chitosan is that over 50% of the glucosamine units are deacetylated. Chitosan has been shown to have favorable properties for wound healing and has been investigated in wound dressings (Rossi et al., 2007; Takei et al., 2012). Moreover, due to its biocompatible properties (Baldrick, 2010) and the ease to add properties by derivatisation, chitosan has been investigated for pharmaceutical and biomedical applications (Alves and Mano, 2008). With the aim of forming nanoparticles, a cationic chitosan derivative was prepared (see Fig. 1) through trimethylation of the backbone. Moreover, carboxymethyl groups were conjugated to the backbone to provide grafting sites for the RGD-containing peptides, Gly-Arg-Gly-Asn-Ser (GRGDS). To be able to compare the impact of the RGD-moiety, a control polymer was synthesized with inverted inactive sequence of GRGDS, Ser-Asn-Gly-Arg-Gly (SDGRG).

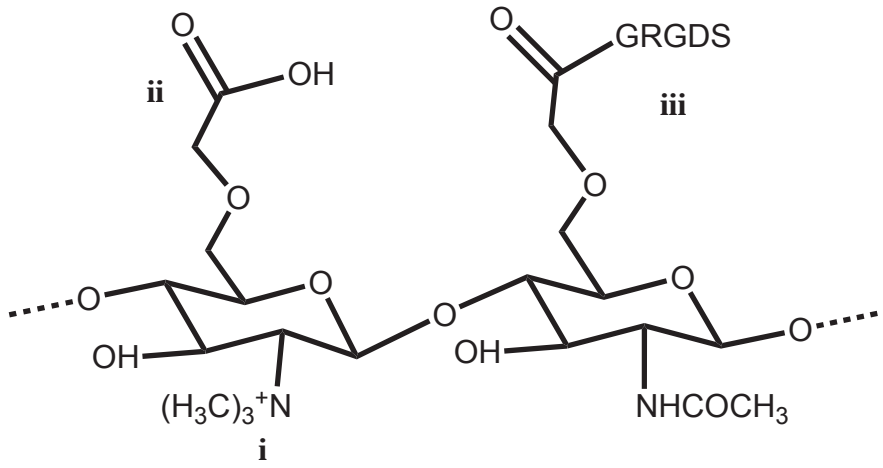


Figure 1. Structure of GRGDS-CM-TMC. The chitosan backbone is functionalized by (i) a trimethylation at the amine group for increased solubility and charge for complexation, (ii) carboxymethyl groups to facilitate the grafting of peptides and (iii) the amino acid sequence GRGDS.

The interaction between GRGDS-CM-TMC and HaCaT were evaluated *in vitro* with HaCaT-cells, an immortalized cell line of keratinocytes (Boukamp et al., 1988). Aspects such as biocompatibility, initial cell adhesion and cell morphology after culture at different time-points were investigated.

2. Material and methods

2.1 Cell culture

An immortalized cell line of human adult keratinocytes, HaCaT was used for all the *in vitro* assays. All cell culture media and additives were bought from PAN Biotech GmbH (Aidenbach, Germany). The HDF were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, 10% fetal calf serum and 1% penicillin/streptomycin. The cells were split at sub-confluency (80%) with trypsin/EDTA diluted in phosphate buffered saline (PBS).

2.2 Cell viability and proliferation assays

The influence of GRGDS- and SDGRG-CM-TMC on cell viability and the proliferation of the HaCaT were assessed using an XTT-cell proliferation kit 2 (Roche, Basel, Switzerland). For the cell viability, the cells were seeded in a 96-well plate (Costar,

Corning, Amsterdam, The Netherlands) at an initial concentration of 15 x 10^3 cells/well. After 24 h of culture, the medium was changed to fresh medium containing indicated concentrations of GRGDS- or SDGRG-CM-TMC, respectively. The cells were cultured in the presence of the polymers for 24 hours before removing the medium and adding XTT-reagent. For the proliferation assays, the cells were plated onto coatings of GRGDS- and SDGRG-CM-TMC at a concentration of 20 x 10^3 cells/well. The cells were grown on the coatings for 24 h before assessing the cell number by adding XTT-reagent. The absorbance was measured at a wavelength of 490 nm after 6 h with a Tecan plate reader (Tecan group Ltd., Männedorf, Switzerland) and the cell viability (%) was expressed in percentage relative to a control group of HDF cultured in the absence of polymer.

2.3 Adhesion assays

96-well plates (Corning) were coated with GRGDS- or SDGRG-CM-TMC at indicated concentrations by overnight adsorption at 4˚C. The wells were subsequently saturated with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1h at ambient temperature. The HaCaT were detached and rinsed in serum-free DMEM before being seeded at a density of 5 x 10^4 cells/well. After 2 h of incubation (37˚C, 5% CO_2), the non-adhered cells were removed by washing with PBS and the attached cells were fixed for 1 hour with a solution of 1% glutaraldehyde (Sigma-Aldrich) in PBS. To evaluate the level of adhesion, the cells were stained for 1 hour with a solution of 0.1 % crystal violet. After drying, the level of adhesion was determined by solubilizing the dye in 1% acetic acid containing 0.1% Triton-X 100 and measuring the optical density at a wavelength of 570 nm with a Tecan plate reader. Cells investigated for their morphology was fixated and stained by immuno

2.4 Culture onto coatings

Drops of GRGDS- and SDGRG-CM-TMC solutions (0.1 mg/ml) were placed on cover glasses and let dry. Once dry, they were placed in 24-well plates (Costar, Corning) and blocked by addition of 1% BSA in PBS for 1 hour. The cells were seeded at a concentration of 20 x 10^4 cells/well and grown on the coatings at different concentrations of fetal calf serum (FCS) for 2-24 h. The non-adhered cells were removed by rinsing with pre-warmed PBS, before the attached cells were fixed and stained with immunofluorescence.

2.5 Immunofluorescence staining

After performed adhesion or culture assays, the cells were fixed in pre-warmed PBS containing 4% paraformaldehyde (Alfa Aesar, Ward Hill, USA), for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 3 min. Samples were then pre-incubated with 1% BSA in PBS for 20 min to prevent non-specific attachment, before being incubated with 6.6 μM phalloidin conjugated to Alexa 488 (Lonza, Basel, Switzerland) in 1 % BSA in PBS for 20 min. After being washed in an excess of PBS, the fixed and stained cells were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenyllindole (DAPI; Vector Laboratories Ltd., Peterborough, UK) before being attached to a microscopic slide. The cell morphology was analyzed with a Zeiss LSM 700 confocal microscope (Carl Zeiss AG, Feldbach, Switzerland) using x10 and x20 objectives.

2.6 Statistical significance

Student's t test (for two samples, assuming equal variance) was used to compare data at a significance level of $p < 0.05$. The results are expressed as mean ± standard deviation.

3. Results

3.1 Cell viability

As an initial approach to the *in vitro* evaluation of GRGDS-CM-TMC, the biocompatibility of the polymer with HaCaT cells was assessed. Cell viability assays were performed by measuring the viability of cells cultured in the presence of polymers in solution during 24 h relative/with respect to the viability of a control group of cells incubated without polymer. The control polymer with an inactive peptide, SDGRG-CM-TMC, was included in the assay. As seen in figure 2, the cell viability decreased to a minimum of 80 % in the presence of the polymer at concentrations 0.2-1 mg/ml. Regardless of the polymer concentration in this concentration range, cell viability remained constant. For a concentration of 0.1 mg/ml, the cell viability was 90 %. There was no significant difference between the two polymers at any concentration (n=3).

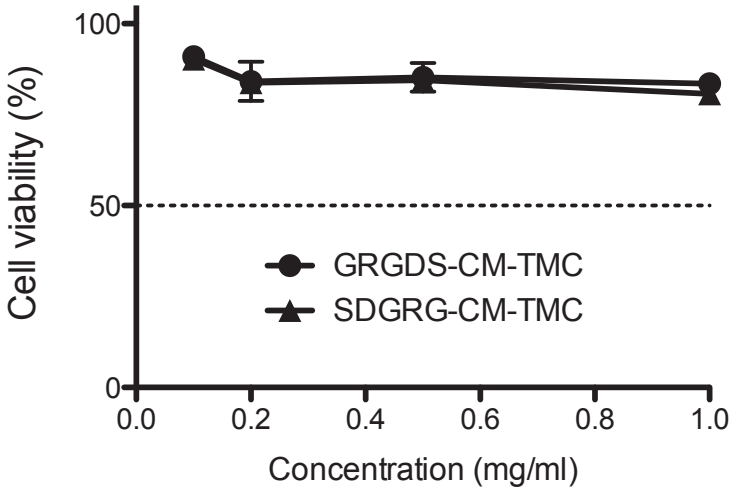


Figure 2. Cell viability of HaCaT after 24 h incubation with GRGDS- and SDGRG-CM-TMC in solution. The cell viability for concentrations ranging between 0.2 and 1 mg/ml were > 80 % and for 0.1 mg/ml 90 % for both polymers (n=3, p>0.05).

3.2 Adhesion assays

To investigate the interaction between HaCaT and the RGD-functionalized polymer, adhesion assays were performed. Briefly, GRGDS-CM-TMC and the inactive control polymer SDGRG-CM-TMC were coated onto 96-well plates. The cells were incubated onto the coatings in plain media. A time-point after 2 hours was chosen to assess cell adherence, however, even at this relatively long incubation-time, the variability in the results was high. Despite several repetitions, no conclusive results were obtained. During certain assays, a dose dependent adhesion was observed, as seen in Fig. 3. However, this adhesion was highly variable and GRGDS-CM-TMC could not be confirmed to have any effect on the level of adhesion by these assays.

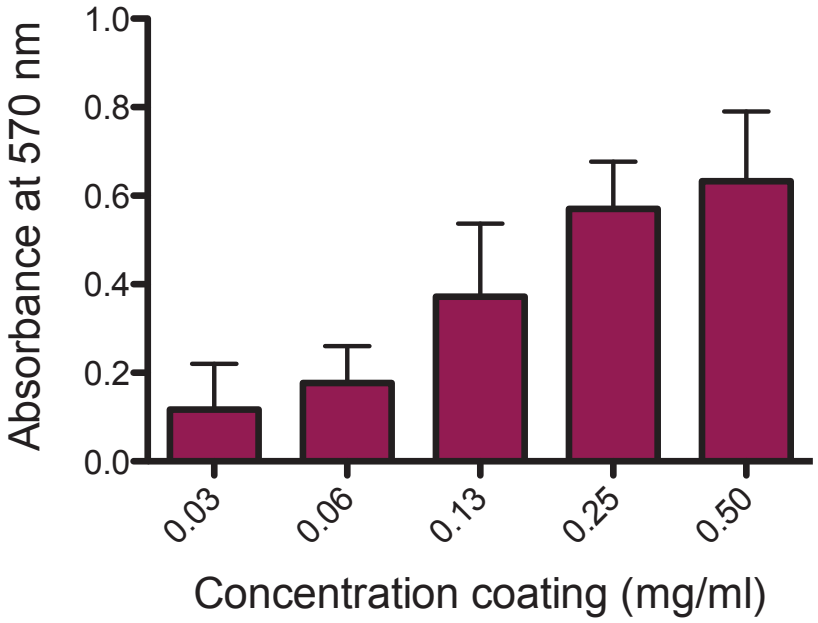


Figure 3. Adhesion of HaCaT onto coatings of GRGDS-CM-TMC of different concentrations.

Changing the approach of investigating the HaCaT-RGD interaction, the morphology of the cells was studied after the adhesion tests. The cells were incubated onto coatings of GRGDS- and SDGRG-CM-TMC and features such as form and spreading of the cells on the two coatings were compared by visualizing the actin fibers. Fig. 4 shows the phenotype of the cells after 2 h incubation on coatings of GRGDS- and SDGRG-CM-TMC in the presence of three different concentrations of FCS (0, 0.5 and 5 %). Different serum concentrations were used to minimize the influence of serum on the cell response.

Even after the relatively long incubation time, the majority of the cells remained spherical, while some of the cells started to spread by stretching out thin filopodia. Comparing the different concentrations of FCS, no difference was observed at this time-point. The cell phenotype on the two coatings was identical. Hence, no impact of the RGD-moieties on the spreading of the HaCaT could be confirmed at this initial stage.

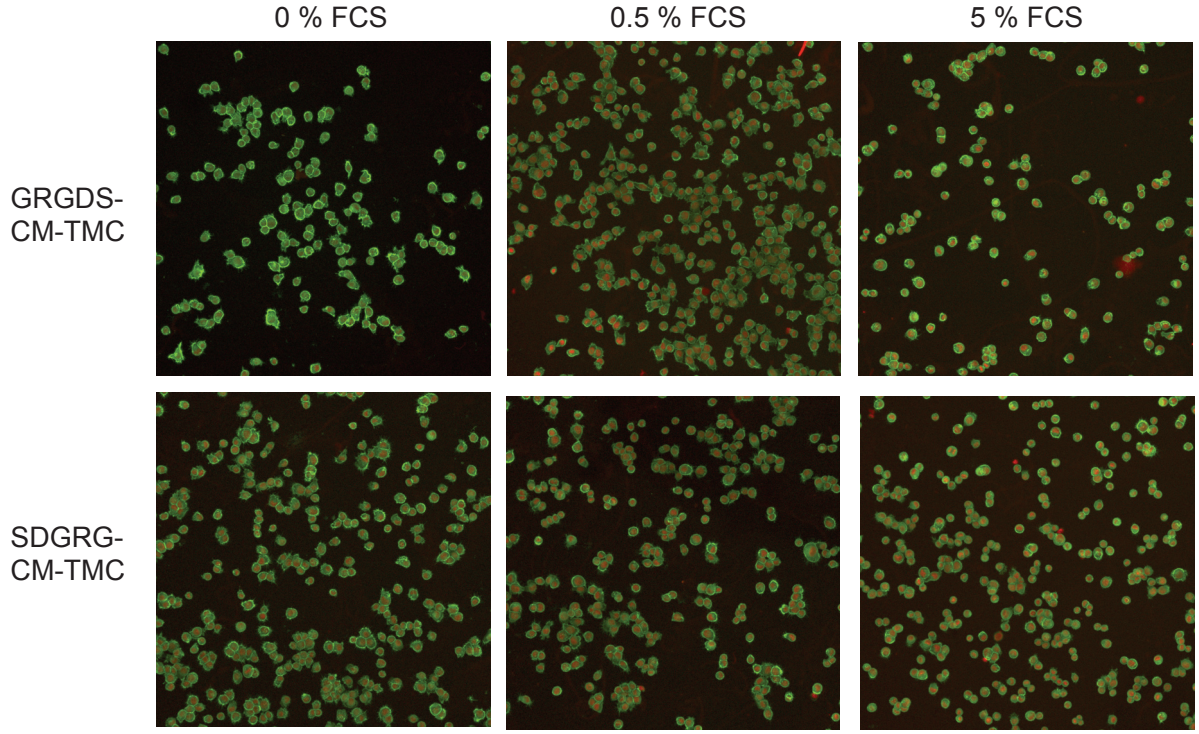


Figure 4. Adhesion assays of HaCaT onto coating of GRGDS- and SDGRG-CM-TMC. The morphology of the cells was investigated after 2 h of incubation with three different concentrations of fetal calf serum (FCS).

3.3 Culture on coatings

To further investigate the impact of GRGDS-CM-TMC on HaCaT, the cells were cultured on coatings of GRGDS-CM-TMC and SDGRG-CM-TMC, respectively, for 8, 12 and 24 h, as shown in Fig. 5. Two different concentrations of FCS were used to evaluate the impact of the GRGDS-moieties on the phenotype (Fig. 5A: 0.5 %, FCS Fig 5B: 5% FCS). Colonies were seen under both conditions at 8 h. At 12 h, the cells incubated with 0.5 % FCS started to be affected by the low supply of nutrients, however, there are no differences in the morphology of the cells on the different coatings. The main difference between the different coatings was found for the cells grown in 5 % FCS for 24 h. The colonies formed were remarkably larger for the cells placed on coatings of GRGDS-CM-TMC compared to the smaller colonies on SDGRG-CM-TMC.

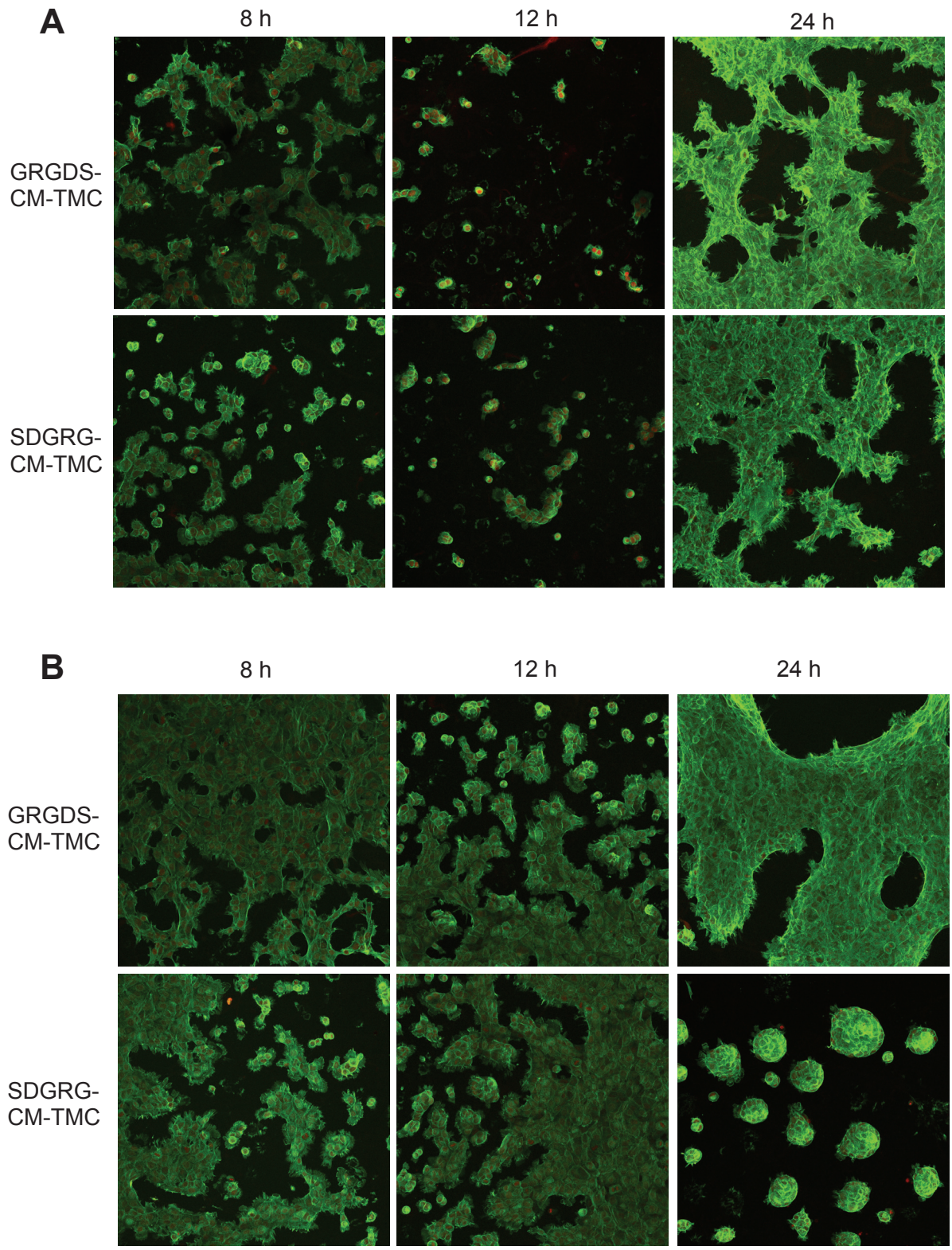


Fig 5. Culture of HaCaT on coatings of GRGDS- and SDGRG-CM-TMC for 8, 12 and 24 h. Two different fetal calf serum (FCS) concentrations were used, A, 0.5 % FCS and B, 5 % FCS.

A hypothesis explaining the difference in the colony formation between the two coatings was an increased proliferation induced by the RGD-containing peptides. To further investigate this possibility, the cell proliferation on the two coatings was assessed and expressed as percent relative to cells cultured in uncoated wells. HaCaT cells were grown on coatings for 24 h and the number of cells was successively estimated measuring the mitochondrial dehydrogenase levels for each condition, as shown in Figure 7. The cell number was > 95 % compared to control wells on coatings formed from 0.05-0.1 mg/ml solutions. For coatings of 0.4 mg/ml, the cell number decreased slightly to 90 %. Comparing the values of GRGDS- and SDGRG-CM-TMC, no significant difference was obtained (n = 5 assays, $p>0.05$), indicating that the difference in colony-formation may be independent of increased proliferation.

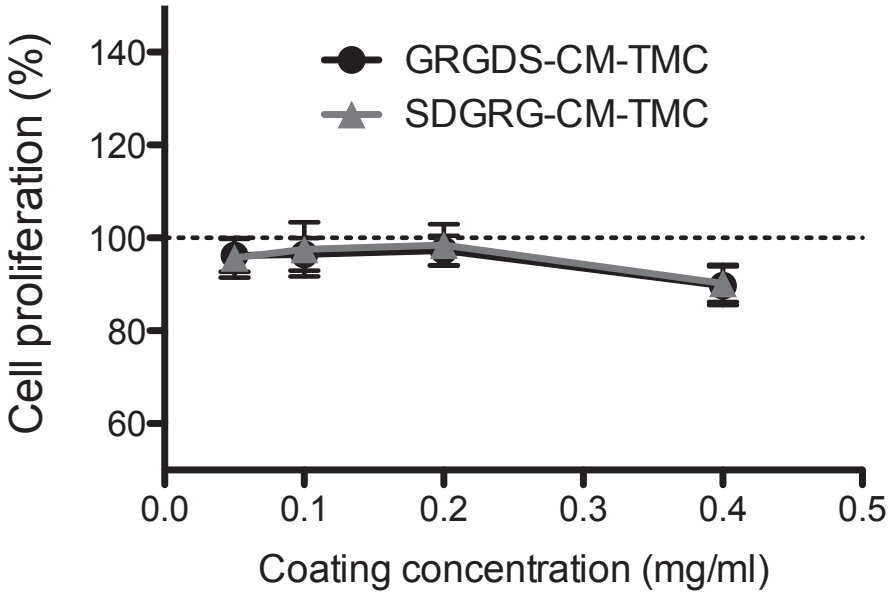


Figure 7. Proliferation on coatings relative to the cell proliferation on tissue culture treated polystyrene. The cell proliferation was > 95 % for concentrations 0.05-0.2 mg/ml and 90 % on the coatings of 0.4 mg/ml. No significant difference between the two different coatings (n=5 assays).

4. Discussion

The main aim of this *in vitro* evaluation was to investigate whether GRGDS-CM-TMC specifically induces cell adhesion and subsequently cell migration in keratinocytes. An immortalized keratinocyte cell line, HaCaT, was used for this purpose.

In a first step of the *in vitro* evaluation, cell viability tests were performed. Applying the GRGDS- and SDGRG-CM-TMC in solution onto the cells, a decrease in the cell viability was seen. A possible explanation for this decrease in cell viability might be an interaction of the positively charged amino-groups in CM-TMC with the tight junctions, opening them up and breaking the integrity of the epithelial sheet. Epithelial cells are connecting cells and need to tightly connected to each other and in drug delivery this mechanism is used to enhance paracellular transport of drugs in mucosal tissues (du Plessis et al., 2010). However, seen the relatively low decrease in cell viability, GRGDS- and SDGRG-CM-TMC are considered to be biocompatible with HaCaT-cells.

During the initial adhesion assays, trends of a dose-dependent adhesion of HaCaT to coatings of increasing concentrations were seen in some of the assays, as shown in Fig. 3. However, frequently no adhesion or no specific trends in the level of adhesion were observed, making it difficult to compare the level of adhesion between GRGDS- and SDGRG-CM-TMC. Hence, the high variability in the results made it impossible to compare and draw any conclusions from the adhesion assays. The occasional dose-dependent adhesion observed in some assays is thought to be non-specific. The interaction between human dermal fibroblasts and GRGDS-CM-TMC was shown to be partly due to non-specific interactions and partially dependent on the GRGDS-moieties of the polymer. The interaction seen with the HaCaT might be due to electrostatic interactions between the polycationic properties of GRGDS-CM-TMC and negatively charged cell membranes.

Seen the inconclusive results assessing the level of adhesion, the morphology of the cells after adhesion assays was examined. After incubating the cells on coatings of GRGDS- and SDGRG-CM-TMC, respectively, the actin fibers and the nuclei were stained with phalloidin conjugated Alexa 488 and DAPI, respectively, and observed by confocal laser scanning microscopy. Comparing the cell morphology on the two coatings at different concentrations of FCS, no difference was observed between the

different coatings. The HaCaT did not spread either on GRGDS- or SDGRG-CM-TMC coatings. Questioning if the interaction was dependent on time of incubation, culture assays over longer periods of time were performed. The morphology at 8, 12 and 24 h time points at different concentrations of FCS was investigated. Despite longer time in contact with the different coatings, no difference in spreading or morphology was observed. The only exception was the colony formation of the cells cultured in 5 % FCS for 24 h, which were remarkably larger then on the coatings of SDGRG-CM-TMC. This observation suggests that some kind of interaction, for example promotion of proliferation or migration, is taking place after longer incubation times.

The formation of these larger colonies may have been the result of an increased cell proliferation. In order to investigate this possibility, the number of HaCaT cultured for 24 h on coatings of GRGDS- and SDGRG-CM-TMC was compared to cells cultured on polystyrene. Compared to the control, the cell proliferation was found to be > 95 % for coatings formed from polymer solutions of a concentration range of 0.2-0.05 mg/ml for both polymers. On the coatings formed from 0.4 mg/ml, a proliferation of 90 % was obtained on both layers. The slight decrease in proliferation on the thicker coating layers might be attributed to less anchored polymer chains and hence less stable adhesion of the cells. More importantly, no difference in the proliferation was seen between the two polymers and the difference in morphology remains unclear. Further investigations are needed to elucidate the possible impact of the peptide.

To summarize the assays performed herein, GRGDS-CM-TMC was shown to be biocompatible to HaCaT. Comparing the cell adhesion and the cell morphology of HaCaT plated onto coatings of GRGDS- and SDGRG-CM-TMC, no difference was observed. Despite the numerous integrins recognizing RGD, no typical signs of adhesion, such as increased level of adhesion or spreading of the cells, were obtained in the assays performed. Several explanations of these results are possible:

i. There is an interaction but RGD-concentration on the polymer might be to low to induce any detectable response.
ii. The interaction takes time to develop and start to have an effect only after 24 h.
iii. Weak binding between the ligand and the receptor due to insufficient presentation of the peptide.
iv. HaCaT do not interact with the RGD-sequence due to lack of expression of receptors recognizing RGD.

Performing *in vitro* assays with human dermal fibroblasts (Hansson et al., 2012), the RGD-peptides were confirmed to be properly presented to interact with cell surface receptors, and to not have been inactivated during the conjugation process. The concentration of RGD peptide used in these experiments, ~60 nmol/mg polymer was sufficient to induce increased adhesion and spreading. However, it cannot be excluded that higher levels of RGD-peptides might be needed for interaction with keratinocytes.

There are remarkably few publications available reporting on a favorable interaction between RGD peptides and keratinocytes. Kim et al. (1992) reported an increased cell migration of normal human keratinocytes on a substrate of RGD. Furthermore, Wang et al. (2006) reported an increased adhesion of normal human keratinocytes to a surface coated with RGD-conjugated fusion proteins. There are also a handful of articles demonstrating an effect of RGD on the epithelialization process applying matrices of collagen/chondroitin sulfate functionalized with RGD-peptides *in vitro* (Cooper et al., 1990; Grzesiak et al., 1997) and of RGD-hyaluronan matrices *in vitro* (Mertz et al., 1996) and *in vivo* (Cooper et al., 1996; Mertz et al., 1996).

According to our assays RGD is not an appropriate adhesion peptide to use for inducing cell adhesion in HaCaT cells. Theses results are supported by recent publications investigating the interaction between HaCaT and biomaterials functionalized with different ECM-derived adhesion peptides. According to these reports, HaCaT not bind to either electrospun fibers (Grafahrend et al., 2010) or polyethyleneglycol (PEG) -stars (Salber et al., 2007) functionalized with RGD-containing peptides. The main integrin receptor involved in keratinocyte migration on

fibronectin is $\alpha 5\beta 1$. This specific receptor is known to preferentially bind to fibronectin or larger fragments of fibronectin, encompassing the 9th and 10th type III module, due to its requirement of the synergy site PHRSN for optimal binding (Aota et al., 1994; Obara et al., 1988). Although keratinocytes express several other RGD-recognizing integrins (Margadant et al., 2010), the expression might be very weak, or not sufficient to induce adhesion and spreading.

Further assays (e.g., migration assays, assays using normal human keratinocytes) and modifications (e.g., increase in RGD-concentration) of the polymer GRGDS-CM-TMC will be needed to investigate the keratinocyte-HaCaT interaction. Other strategies including RGD-peptides are also conceivable, for example to use mixtures of ECM-derived adhesion peptides. It has been shown that mixing RGD-peptides with peptides from collagen (Grafahrend et al., 2010) and laminin (Salber et al., 2007), increases the level of adhesion synergistically compared to the collagen- and laminin-peptides alone. These results indicate that RGD alone might not be sufficient to induce adhesion and spreading, although it might have favorable effects in combination with other peptides or proteins

Conclusion

The aim of this study was to investigate the interaction between a polymer functionalized with RGD-moieties and keratinocytes. The expected response of increased adhesion and spreading of the cells was not observed. Comparing the morphology of cells plated on GRGDS-CM-TMC and an inactive control coating, no difference in phenotype was observed. Hence we conclude that our polymer is not sufficient to induce RGD-mediated attachment and spreading in HaCaT. Further assays are needed to investigate the RGD-HaCaT interaction. New approaches might be applied, for example by using a mixture of adhesion peptides.

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Supplementary figures

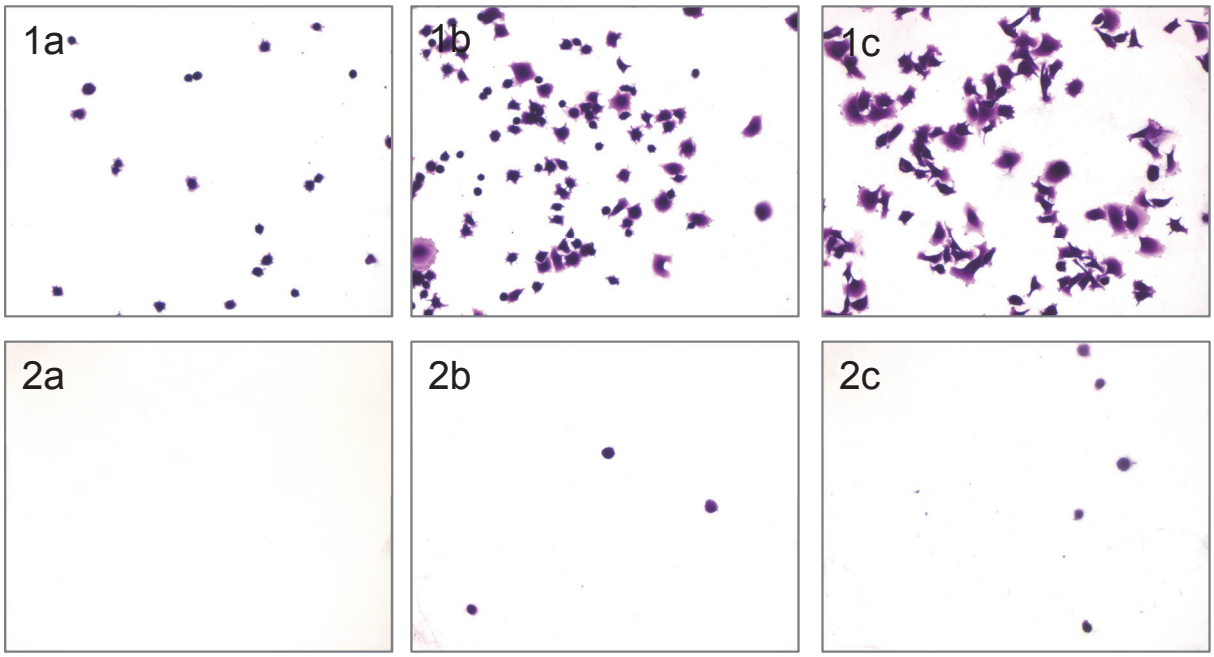


Figure S1. Spreading of HaCaT on coated fibronectin (1) and on a blank control surface (2). As a control to coatings of GRGDS-CM-TMC, the morphology of HaCaT on 50 µg/ml was investigated to compare the results obtained with GRGDS-CM-TMC to optimal conditions. In order to visualize the phenotype, the cells were fixed and stained with crystal violet after 0.5 h (a), 1 h (b) and 2 h (c) incubation in serum-free media.

Summary and conclusion

The aim of the work presented in this thesis, was to develop functionalized nanoparticles with the ability to induce adhesion and migration in normal human keratinocytes. Using particulate systems to promote and support cell adhesion and migration in epidermal restoration is a novel approach of tissue engineering. Nanoparticles functionalized with the adhesion promoting sequence Arg-Gly-Asp (RGD) were aimed to be applied as a substrate for keratinocytes when the normal wound healing process is impaired or not sufficient.

In Chapter 1, a bibliographic review is given of the application of RGD-peptides in combination with biomaterials in dermal and epidermal tissue reconstruction. Hitherto, RGD has been combined with natural polymers such as proteins, polysaccharides and glycosaminoglycans, as well as synthetic polymers as scaffolds in dermal substitutes. For fibroblasts, RGD was clearly promoting adhesion and migration *in vitro*. For keratinocytes, the interaction was more difficult to define due to conflicting reports. *In vivo,* several positive observations have been made, however, the clinical effect of RGD-conjugated biomaterials remains to be confirmed for both fibroblasts and keratinocytes.

In order to further investigate the role of biomaterials grafted with adhesion peptides, an RGD-functionalized chitosan derivative was synthesized, as described in Chapter 2. Briefly, a positive charge was added to the backbone of chitosan to confer increased water solubility and facilitate complexation with polyelectrolytes. Moreover, carboxymethyl groups were added to the derivative to facilitate the grafting of adhesion peptides to the polymer. In a final step, an RGD-containing peptide, Gly-Arg-Gly-Asp-Ser (GRGDS) was grafted to the chitosan derivative, giving the derivative GRGDS-carboxymethyl-trimethyl chitosan (GRGDS-CM-TMC). The bioactivity of the new polymer was assessed through *in vitro* assays with human dermal fibroblasts (HDF). HDF were chosen as a suitable cell model due to their documented interaction with GRGDS-moieties through $\alpha V\beta 3$ integrin receptors. The bioactivity of the novel polymer was confirmed *in vitro* by an increased cell adhesion

and spreading of the HDF when in contact with the GRGDS-polymer. This response was shown to be specific to interaction with GRGDS-moieties. Hence it was concluded that a polymer with ability to induce adhesion and spreading was successfully developed.

The polymer GRGDS-CM-TMC was designed to be able to form nanoparticles by complexation. Chondroitin sulfate, a component of the extracellular matrix (ECM), was chosen as a suitable counterion to prepare nanoparticles, as shown in Chapter 3. The particles formed were shown to be of spherical shape, in the size range of 150-200 nm with a narrow size distribution. In physiological medium the particles remained intact but swelled significantly. The bioactivity of the particulate system was assessed *in vitro* using HDF. The exposure of GRGDS peptides on the particle surface was shown to increase the level of adhesion in addition to induce spreading of the cells. With these observations it was concluded that a functional particulate system was developed with sufficient mechanical strength to support cell adhesion in its hydrated form and an appropriate exposure of the bioactive GRGDS peptides was obtained.

As the last step of the development and evaluation of GRGDS-CM-TMC and particles formed thereof, *in vitro* assays with an immortalized keratinocyte cell line, HaCaT, were performed, as presented in Chapter 4. The polymer was demonstrated to be biocompatible with the keratinocytes through cell viability assays. However, when assessing the bioactivity of the GRGDS-functionalized polymer, no clear cell response was observed. The level of cell adhesion was highly varying and no conclusion could be drawn. When investigating the morphology of the cells, no spreading could be observed at an initial stage. Neither at later time points any difference in phenotype due to GRGDS-exposure was seen. From these observations the conclusion was drawn that either the concentrations of RGD-peptide was not sufficient or GRGDS is not an appropriate ligand for the integrins expressed by keratinocytes.

To summarize, a GRGDS-functionalized chitosan derivative was successfully developed. The bioactivity of GRGDS was preserved during synthesis and the novel polymer showed excellent properties for particles formation. Particles in the nanorange with a narrow size distribution were formed through complexation

between GRGDS-CM-TMC and chondroitin sulfate. The particles showed to be stable in physiological medium but increased significantly in size due to swelling. Despite the swelling, the particles showed mechanical strength suitable to support cell attachment. Moreover, the particles were shown to be bioactive as the increased cell adhesion and spreading of cells. However, when investigating GRGDS-CM-TMC with keratinocytes, no clear cell response was confirmed. The cell adhesion was highly variable and no spreading of the cells was induced by the GRGDS-moieties.

Hence, it was concluded that a functional particulate system was successfully developed, but the interaction with keratinocytes remains unclear. Further assays are needed to elucidate whether GRGDS is a suitable cell adhesion peptide for keratinocytes or if other options, such as adhesion peptides derived form other ECM proteins should be applied or mixtures of adhesion peptides should be considered for the preparation of a particulate system. Further *in vitro* assays are needed in order to optimize the particulate system before engaging in assessment *in vivo*.

Résumé et conclusion

L'objectif du travail présenté dans cette thèse était de développer des nanoparticules capables d'induire l'adhésion et la migration de kératinocytes humains. L'usage de systèmes particulaires pour favoriser l'adhésion et la migration cellulaire constitue une technique novatrice de réparation tissulaire. Les nanoparticules rendues fonctionnelles par le greffage d'une séquence Arg-Gly-Asp (RGD) agissent comme un substrat pour les kératinocytes lorsque le processus de cicatrisation est altéré ou insuffisant.

Dans le premier chapitre, une revue bibliographique présente les différentes applications du peptide RGD dans les phénomènes de réparation cutanée. Jusqu'à présent, le RGD a été combiné avec des polymères naturels tels que des protéines, des polysaccharides et des glycosaminoglycanes, mais également avec des polymères synthétiques comme matrice pour les substituts dermiques. *In vitro*, le RGD agit clairement comme un facteur d'adhésion et de migration sur le fibroblaste. En revanche son action sur le kératinocyte est plus délicate à définir et les données de la littérature sont à ce sujet contradictoires. *In vivo*, les effets cliniques de la combinaison RGD-biomatériaux sur les fibroblastes et les kératinocytes restent à confirmer.

Dans le but de caractériser le rôle des biomatériaux couplés à des peptides d'adhésion, un chitosan fonctionnalisé avec RGD a été synthétisé selon une technique décrite dans le chapitre 2. Une charge positive a été ajoutée au chitosan pour accroître sa solubilité dans l'eau et faciliter la formation de nanocomplexes polyélectrolytiques. De plus, des radicaux carboxymethyl ont été également ajoutés pour faciliter le greffage de peptides d'adhésion au polymère. Enfin, le peptide Gly-Arg-Gly-Asp-Ser (GRGDS) contenant le RGD a été greffé au dérivé de chitosan pour former le GRGDS-carboxyméthyle-triméthyle-chitosan. L'activité de ce nouveau polymère a été testée *in vitro* sur des fibroblastes dermiques humains (FDH). Ces FDH ont été choisi comme modèles cellulaires en raison de leurs possibilités d'interactions avec le GRGDS via le récepteur intégrine $\alpha V\beta 3$. L'activité biologique

du nouveau polymère a été confirmée *in vitro* par sa capacité à induire l'adhésion et l'étalement des FDH. Cette activité est spécifique à l'interaction avec les séquences peptidiques GRGDS. Ainsi, un polymère capable d'induire l'adhésion et l'étalement des FDH à été développé avec succès.

Le polymère GRGDS-CM-TMC a été conçu pour être capable de former des nanoparticules par complexation. Le sulfate de chondroïtine, un composant de la matrice extra-cellulaire (MEC), a été choisi comme un contre-ion adéquat pour la préparation des nanoparticules comme décrit dans le chapitre 3. Les particules ainsi formées sont de forme sphérique avec une distribution étroite de taille entre 150 et 200 nm. En milieu physiologique, ces particules restent intactes mais gonflent de façon significative. L'activité biologique de ces particules a été évaluée *in vitro* à l'aide des FDH. Les peptides GRGDS présents sur la surface des particules ont augmenté le taux d'adhésion et induit une migration cellulaire. Un système particulaire fonctionnel a ainsi été développé, dont la forme hydratée possède une stabilité mécanique suffisante pour promouvoir l'adhésion cellulaire au travers d'une présentation appropriée de peptides GRGDS bioactifs.

Dans la dernière étape du développement et de l'évaluation du GRGDS-CM-TM et des particules, des tests *in vitro* avec la lignée cellulaire HaCaT de kératinocytes immortalisés ont été entrepris, décrits dans le chapitre 4. Des essais de viabilités cellulaires ont montré la biocompatibilité du polymère avec les kératinocytes. Cependant, l'évaluation de l'activité biologique du polymère GRGDS n'a pas révélé de réponse cellulaire claire en raison du niveau d'adhésion cellulaire très variable. Concernant la morphologie cellulaire, aucun étalement n'a été observé durant la phase initiale, ni aucune différence de phénotype à des temps d'exposition plus long au GRGDS. De ces observations, il était conclu que soit la concentration en peptide-GRGDS était insuffisante, soit que le GRGDS n'était pas un bon ligand pour les intégrines exprimées par les kératinocytes.

Pour résumer, un chitosan dérivé avec des peptides GRGDS a été développé avec succès. L'activité biologique du GRGDS a été préservée durant la synthèse et le nouveau polymère a montré d'excellentes propriétés pour la formation de particules. Des particules de l'ordre du nanomètre, avec une distribution de taille étroite ont pu être formées par complexation entre le GRGDS-CM-TMC et le sulfate de

chondroïtine. Les particules sont stables dans le milieu physiologique malgré un accroissement de leur taille. En dépit de ce gonflement, ces particules se sont révélées suffisamment stable pour permettre une liaison avec les cellules. Cependant, concernant l'interaction entre le GRGDS-CM-TMC et les kératinocytes, l'adhésion cellulaire s'est révélée très variable et aucun étalement cellulaire ne fut observé. Le système nanoparticulaire développé est donc capable d'interagir clairement avec les FDH, mais son interaction avec les kératinocytes est encore imparfaitement comprise. Des investigations supplémentaires sont nécessaires pour préciser si le GRGDS est un peptide d'adhésion cellulaire adéquate pour les kératinocytes, ou si d'autres peptides d'adhésions dérivés de la MEC doivent être envisagés pour la préparation de systèmes particulaires. D'autres études *in vitro* doivent être réalisées pour optimiser le système particulaire avant d'envisager des évaluations *in vivo*.

Abbreviations

AAA	Amino acid analysis
BSA	Bovine serum albumine
CM-TM-chitosan	Carboxymethyl-trimethylchitosan
CM-TMC	Carboxymethyl-trimethylchitosan
CMC	Carboxymethyl cellulose
CS	Chondroitin sulfate
DAPI	4’,6-diamidino-2-phenylindole
DIC	Differential interference contrast microscopy
DMEM	Dulbecco’s Modifieds Eagle’s Medium
DS	Degree of substitution
ECM	Extracellular matrix
EDC	N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FN	Fibronectine
FTIR	Fourier transform infrared spectroscopy
GAG	Glycosaminoglycan
GF	Growth factor
HA	Hyaluronic acid
HDF	Human dermal fibroblasts
HF	Hair follicle
IGF	Insulin growth factor
KGF	Keratinocyte growth factor
LAP	Latency-associated protein
LN	Laminin
NHK	Normal human keratinocytes
NHS	N-hydroxy succinimide
NMP	Methylpyrrolidone
NMR	Nuclear magnetic resonance

NP	Nanoparticles
PBS	Phosphate buffered saline
PCS	Photon correlation spectroscopy
PDI	Polydispersity index
PEC	Polyelectrolytic complexe
PEG	Polyethylene glycol
PHSRN	Prolin-Histamin-Serin-Arginin-Arspatic acid
PLGA	Poly (lactic-co-glycolic acid)
PVA	Polyvinyl alcohol
RGD	Arginine-Glycin-Aspargin
SEM	Scanning electron microscopy
TM-chitosan	Trimethyl chitosan
TMC	Trimethyl chitosan
TN	Tenascin
TSP	Trimethylsilyl propionic acid-d4 sodium salt
VN	Vitronectin