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Interaction studies of chemokines and chemokine mutants

MASTER THESIS

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Zusammenfassung

Chemokine gehören zu einer Familie kleiner chemotaktischer Proteine, die an verschiedensten biologischen Prozessen beteiligt sind. Neben Interaktionen mit ihren GPCRs und GAG Co-Rezeptoren zur Ausübung der biologischen Funktion interagieren sie außerdem mit anderen Chemokinen. In diversen Studien wurde die signifikante Auswirkung homo- und heterophiler Interaktionen auf die Aktivität der Chemokine festgestellt. In dieser Studie wurden die Oligomerisierungs-Charakteristiken von IL-8, MCP-1 und SDF-1, sowie deren Decoys PA401, PA910 und PA1011, entwickelt von ProtAffin Biotechnologie AG, untersucht. Die Chemokine wurden mit FITC markiert und mithilfe verschiedener Verfahren analysiert wobei sich IFT als geeignetstes Verfahren erwies. Anhand von SPR wurde die Auswirkung der FITC Markierung auf die Bindung mit HS untersucht. Die Resultate zeigen, dass alle getesteten Wildtyp und Decoy Chemokine Heterooligomere formen. Die Reaktivität der Chemokine wiederum hängt davon ab, ob sie als Ligand oder markiertes Protein eingesetzt werden, was zu der Annahme führte, dass FITC Einfluss auf das Bindungsverhalten hat. Auch wurden verschiedene Oligomerisierungs Charakteristiken zwischen Wildtypen und Decoys gefunden; zusammengefasst zeigen die Mutanten eine leicht verringerte Interaktion verglichen mit den entsprechenden Wildtypen. Von den getesteten Chemokinen interagieren MCP-1 und die entsprechende Mutante PA910 mit mehreren FITC markierten Chemokinen, FITC PA401 nur mit MCP-1. Mittels SPR wurde anhand des K_D Wertes der Einfluss von FITC auf die Bindungsaffinität nachgewiesen. Markierte Chemokine weisen eine stark verringerte Affinität zu HS auf. Ein Eingreifen nicht nur direkt auf die Chemokinaktivität sondern auch auf die Chemokininteraktion könnte Potential für therapeutische Anwendungen bieten.

Abstract

Chemokines comprise a family of small, chemoattractant proteins involved in many physiological processes. Despite interacting with their GPCRs and GAG co-receptors to exert their biological function they also interact with other chemokines. Different studies reveal the significant affection of the biological activity by homo- and heterophilic interaction. In this study we determined the oligomerization characteristics of IL-8, MCP-1 and SDF-1 as well as their decoys PA401, PA910 and PA1011, developed by ProtAffin Biotechnology AG. The chemokines were labeled with FITC and different approaches were tested whereas IFT has shown to work best. SPR was performed to investigate the impact of FITC on interaction, exemplary with HS. The results indicate that all tested wild type and decoy chemokines form heterooligomers. Reactivity was found to depend on whether the respective chemokines was used as ligand or as labeled protein, which is assumed as an impact of FITC labeling. Also different oligomerization characteristics of wild types and mutant chemokines were observed; summarized decoys show a slightly impaired oligomerization behavior compared to the respective wild type. Of all tested chemokines, non-labeled wt MCP-1 and its decoy PA910 have shown to interact to a greater extent with labeled chemokines whereas PA401 interacted only with wt MCP-1. SPR further confirmed the impact of FITC as the assessed K_D values indicate a significant difference related to the binding affinity towards HS of labeled and non-labeled chemokines. Interfering not only chemokine action but also heterophilic interaction might offer potential targets in therapeutic approaches.

Abbreviations

APS	Ammonium persulfate
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GPCR	G-protein coupled receptor
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
IL-8	Interleukin 8
IFT	Isothermal fluorescence titration
K_D	Dissociation constant
MCP-1	Monocyte-chemoattractant protein 1
PBS	Phosphate buffered saline
PAA	Polyacrylamid
RT	Room temperature
RU	Response unit
SDF-1	Stromal cell-derived factor-1
SPR	Surface plasmon resonance
TEMED	Tetramethylethylenediamine

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Objective of work

Chemokines are known to be involved in various pathophysiological processes by interaction with their GPCRs or GAG co-receptors. Their function might be influenced also by their oligomerization characteristics. The aim of this work is to figure out which of the applied chemokines oligomerize in a homo- or heterophilic way in solution and upon binding to heparin. Furthermore oligomerization characteristics of some of ProtAffins decoy chemokines should be compared to wild types. To perform this, interaction of FITC labeled and non-labeled chemokines is analyzed using Gel Shift Assay, Westernblot and IFT. To figure out the impact of FITC, the binding affinity towards HS of labeled chemokines will be compared to non-labeled ones by SPR. This research is performed to get a better insight in chemokine-mediated processes, wherefore determination of chemokine oligomerization might be an essential step.

1 Introduction

1.1 Chemokines

Chemokines are small (6 to 12 kDa in their monomeric form) inducible proteins, secreted by a number of cell types. Derived from "chemoattractant cytokines", they are distinguished due to their action on the G-protein-coupled, seven-transmembrane spanning receptor family (GPCR) where they spark changes in the cytoskeleton and adhesive interaction with the surfaces of cells and the extracellular matrix to induce motion. [1–4] Cellular migration is induced through the activation of GPCR by chemokines, wheras the direction of chemotaxis is guided by a concentration gradient as the secreted chemokines are immobilized along the endothelial surface [5]. Dimerization or oligomerization as well as GAG-interaction are crucial for the activity of chemokines *in vivo*. [6]

In their major role of mediating cell directional migration of basically leucocytes they play an important role in the development and maintenance of both the innate and adaptive immune system as well as in inflammatory processes, autoimmune diseases and cancer. [1,7,8] Besides recruitment, the function of this basic, chemotactic cytokines involves also cellular activation, differentiation, wound repair and angiogenesis. [9]

To date, approximately 50 different human chemokines have been identified that show a 20 – 90 percent sequence homology in their amino acid sequence. [10,11]

1.1.1 Classification

Depending on their function, chemokines can be subdivided into two classes: Homing chemokines are constitutively expressed in ascertain areas of lymphoid tissue, they allocate lymphocytes and dendritic cells within the immune system and are responsible for routine leucocyte trafficking. Inflammatory or inducible chemokines on the other hand are produced by

several cell types and immigrating leucocytes as a response to physiological stress and inflammation. Their main function is the recruitment of leucocytes. [1,4]

Apart from their function, chemokines can be classified into four subfamilies due to their primary structure, based on the relative position of the first two of their four highly conserved cysteine residues near the N-terminus: CXC, CC, C and CX_3C. In the largest subfamilies CXC (α) and CC (β), the first cysteine residues are either separated by a single amino acid, as this is the case for α-chemokines or adjacent in case of β-chemokines. Two essential disulfide bridges Cys1-Cys3 and Cys2-Cys4 are formed in all chemokines. [1,7,12–14]

A-chemokines can be further subdivided into those being angiogenic because of a comprised ELR motif, a glutamic acid–leucine–arginine sequence in the N-terminus, (ELR^+ chemokines, whereof IL-8 is the prototype) and those that lack the motif and act on lymphocytes, the angiostatic chemokines (ELR^-). ELR^+ chemokines bind to the CXCR2 receptor and are angiogenic whereas ELR^- chemokines bind to CXCR3 and are angiostatic. [1,6,15] An exception is CXCL12, which binds to CXCR4 and CXCR7.[6] Also β-chemokines can be subdivided into two groups: The eotaxin monocyte-chemoattractant protein family of which MCP-1 is the best characterized one and the residual β-chemokines. B-chemokines attract with alterable specificity monocytes, eosinophiles, basophiles and lymphocytes but do not act on neutrophils. Lymphoactin with only two cysteine residues and fractalkine with the first two residues separated from the third by three amino acids are the only known examples of C and CX_3C chemokines, both inducing chemotaxis on T-cells and natural killer cells. [1,13,15,16]

1.1.2 Structure

Chemokine monomers generally have a common monomeric fold, containing an unstructured N-terminal domain, which is an important signaling domain, followed by an irregular loop, a 3^{10} helix, a β-sheet composed of three anti-parallel β-strands and a C-terminal α -helix. [17–19]

Four invariant, disulfide bond forming cysteine residues define the highly variable primary structure of chemokines, which is also basis for their classification. [11,20]

The secondary structure elements vary in length. They consist of an elongated N-terminus that antecedes the first cysteine and has no distinct structural character. A loop of approximately ten residues that is often accomplished by a 3^{10} helix follows the first two cysteines. The N-loop between the second cysteine and the 3^{10} helix plays an important functional role. The single-turn 3^{10} helix is followed by three anti-parallel β-strands and a C-terminal α-helix shown in Figure 1.

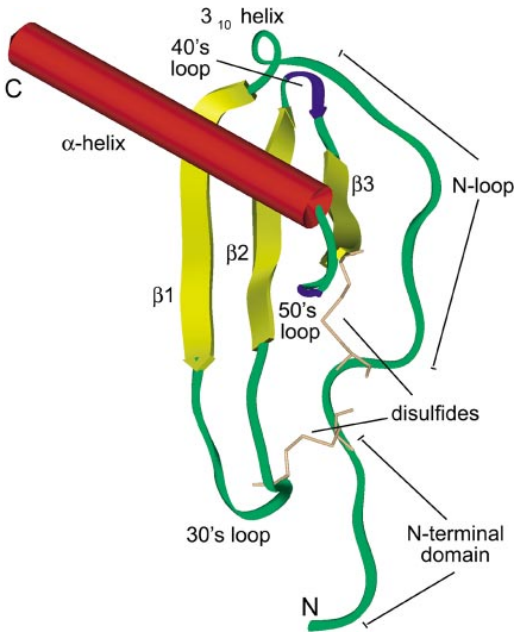


Figure 1 IL-8 monomer with the secondary structure elements described. **[**E. Fernandez et al. *Annu. Rev. Pharmacol. Toxicol.,* 2002**]**

30s, 40s and 50s loops, the numbering of residues in the mature protein, connect each secondary structural unit and carry furthermore the latter two cysteines. The flexibility of the N-terminus is determined by the first two cysteines, which is, despite the two cysteines, greater than in other regions of the protein and might play a role in chemokine receptor binding and activation. [20]

Although the primary structure is highly variable, the tertiary structure of all chemokines shows a highly conserved monomeric structural fold regardless of class, function or receptor binding. The presence of disulfide bonds empowers the primary sequence of chemokines with a low sequence homology to adapt similar tertiary folds. The tertiary structure generally comprises a core domain with a conformationally disordered N-terminus, a long irregular loop proceeded by a 3^{10} helix, a three-stranded anti-parallel β-sheet and a C-terminal α –helix that is packed against the core β-sheet domain. [10,11,21–23] The C-terminal α –helix can define dimerization or GAG binding. [3]

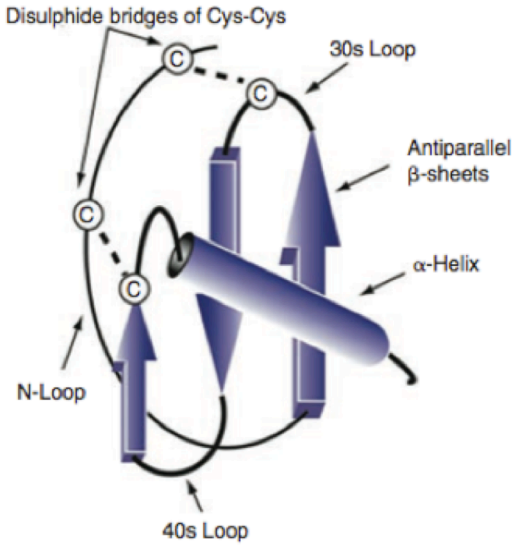


Figure 2 Three dimensional structure of MCP-1, showing the α-helix and β-sheet as well as 30s and 40s loops. [Satish L. Deshmane et al. JOURNAL OF INTERFERON & CYTOKINE RESEARCH, Volume 29, Number 6, 2009]

The β-strands are linked to each other by a flexible 30s or 40s loop, shown in Figure 2. The 30s loop is important for the activity of several chemokines. Hydrophobic interaction between one site of the α–helix and a part of the β-sheet as well as two disulfides stabilize the core of the structure. [20]

Despite the similar tertiary structure, CC, CX_3C and CXC chemokines differ in their quaternary arrangement of dimers, which could explain the specific binding and activation of receptors within a subfamily. While dimers of the distinct subfamilies are different in their

quaternary topology, the monomeric form of chemokines is similar. CC-chemokines dimerize in an elongated form through their N-termini whereas CXC-chemokines dimerize to a compact globular structure with an enlarged β-sheet, exemplary shown in Figure 2. The dimer is formed by the association of two monomers, resulting in two helices packed against a β-sheet. Commonly, CC chemokines associate into enlarged structures whereas CXC and CX_3C chemokines dimerize into a globular form. The only exception is MCP-1, which dimerizes into both, CC- and CXC-like formations. [20,24–26]

Amino acid residues, existent at the distinct inter-subunit interfaces, determine the structure. The different form of dimerization that discriminates CC from CXC chemokines is associated to distinction in the electrostatic surface topology. The first β-sheet of CXC chemokines contains more hydrophobic residues compared to CC chemokines. Therefore they tend to dimerize using the first β-sheet as interface between the monomers, which results in a decreasing exposure of the apolar side chains into the aqueous environment. The dimer interface of CC chemokines is derived by a combination of hydrophobic and electrostatic interactions. [12,20]

If an arrangement of particular residues at a specific monomer interface in a heterodimer is energetically and sterically more favorable than that in the homodimer, monomers of distinct chemokines may be mutually substitutable. [12]

1.1.3 Chemokine Homo- and Heterooligomerization

Chemokines can form dimers or tetramers. Despite the similar monomeric form, CXC and CC chemokines oligomerize in different ways.[12] Many CC chemokines dimerize by the antiparallel organization of N-terminal strands locating the α-helix at the exterior. In comparison, CXC chemokines dimerize through antiparallel organization of the β-sheets by using the interface between the first β-sheets typical for all CXC members.[27]

Apart from dimerization, many chemokines form higher order oligomers upon binding to GAGs or also by themselves. [17] Also association with soluble GAGs induces oligomerization of most chemokines.[5] Interaction with GAGs often favors chemokine oligomerization and the other way round. [23] The stability of these oligomers varies among the different chemokines: MCP-1 changes easily between monomers and dimers, whereas RANTES or MIP form stable large oligomers. [17] Heterooligomerization of RANTES and PF-4 is depicted in Figure 3.

The monomeric form of chemokines has been shown to be necessary for receptor binding to induce cell migration. [17,23] Nevertheless oligomerization might be involved in complex functions

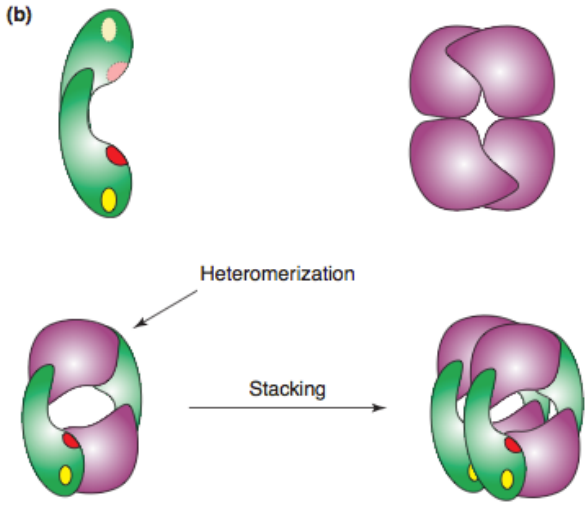


Figure 3 Simple model of RANTES interacting with PF4. The RANTES monomers possibly interact with PF4 monomers to create a "cross over hetero-tetramer". Stacking of the concurrent and adjacent hetero-tetramer might stabilize the complex. [C. Weber and R. Koenen TRENDS in Immunology 27/6 (2006) 268-273]

Nevertheless oligomerization might be involved in complex functions including regulation, haptotactic gradient formation, protection from proteolysis and signaling as well as cell migration. Furthermore oligomerization may also account for functional regulation of chemokines and can promote additional signaling pathways. [23] Heterophilic interactions between chemokines can enhance or suppress the aboriginal function. Moreover a specific chemokine can have different functions depending on the interaction partner and the surrounding milieu.[28] Heterodimerization occurs both within members of a subfamily and between subfamilies.[23] Thirteen chemokines have been identified that perform heterophilic interaction, forming 10 different complexes.[25] The flexibility of the chemokine network can be increased by these interactions, arousing the possibility to regulate and direct leucocytes to inflamed tissue. Heterodimer formation protects chemokines from enzymatic digestion and so stabilizes their activity, as this is the case for MIP-1β and MIP-1 α.[27,28] The biological activity is modulated greatly as a result of heterophilic interactions between CXC and CC chemokines.[12,27] Instancing, the anti-proliferative activity of PF4 towards endothelial cells is increased through heterophilic interactions with IL-8.[28] The conserved monomeric structure enables heterodimerization that is primarily mediated by non-electrostatic interactions. The arrangement of distinct amino acid residues pairs at the dimer interface of the monomers seems to determine thermodynamic stability and choice of dimer type.[12] This is applied especially to CC-CXC mixed heterodimers where the selection of heterodimer types is determined by the placement of specific amino acid residues like positively or negatively charged as well as polar or hydrophobic ones within the β-sheet and the N-terminus. [12]

IL-8 is capable of inducing migration in both forms, monomeric and dimeric. [17] In association with PF4 it forms heterodimers to modulate chemotactic and cell proliferation processes. [29] MIP-1α, PF4, MCP-1 and IL-8 are known to cause synergetic suppression whereas SDF-1 α or GRO- α block the effects of PF4, MCP-1 and IL-8. The presence and activity of GPCR is requisited for the synergy of IL-8 and SDF-1 α. [27]

1.1.4 IL-8

Interleukin-8/CXCL8 is a 8 kDa proinflammatory member of the CXC chemokines, which is known to self-associate as dimers and tetramers, enhanced by the interaction with cell surface GAGs. Secreted by cytokine activated endothelial cells at the side of inflammation, IL-8 is retained at the cell surface through interaction with HSPGs, establishing a local chemokine concentration gradient for the recruitment of neutrophils to the damaged tissue.[29–32] IL-8 can exist reversibly as a monomer or as a dimer, showing a different recruitment profile. [31] Engineered obligate monomers and dimers are reported to still induce cell recruitment while the wild type shows intermediate recruitment characteristics, which leads to the assumption IL-8 exists as a natural equilibrium between both structures. [33] For dimerization, the monomeric β-sheets are extended intermolecular into a six-stranded antiparallel β-sheet. To form tetramers, the β-sheets are cooping the two dimers. [29]

In concentrated solution, IL-8 is a homodimer build up out of two identical subunits. The monomer contains a disordered amino-terminus, stabilized by two disulfide bonds, which is followed by a loop, three antiparallel β-strands and an α -helix extending to the carboxy-terminus. At nanomolar concentration, that represents the physiological level, IL-8 exists as a monomer, which has been shown experimentally is the functional form. [29,31,34] The genes are located on human chromosome 4. Transcription encodes a 99 amino acid precursor protein with a signal sequence that is processed in order to attain a signaling competent mature protein of either 72 amino acids in monocytes and macrophages or 77 amino acids in non-immune cells. [35,36] IL-8 is produced by leukocytic cells as well as somatic cells like endothelial cells and fibroblasts. The production is induced by proinflammatory cytokines as well as bacteria, viruses or environmental factors and is regulated at the level of gene transcription and mRNA stability. The biological activity of IL-8 is mediated by binding to two membrane bound G-protein-coupled receptors, CXCR1 and CXCR2.[34,36]

IL-8 occurs at site of bacterial infection to erase agitating bacteria, as their products can induces IL-8 production in a variety of cell lines *in vitro*. [34] Furthermore it has distinct functions in immune surveillance, inflammation and the enhancement of angiogenesis in tumors by modulating cell proliferation and migration.[37] Angiogenic activity has been reported for all ELR^+ CXC chemokines. IL-8 is a key mediator in neutrophil-mediated acute inflammation, which makes it a potential target for novel therapeutics. [34]

1.1.5 MCP-1

Monocyte chemoattractant protein-1 belongs to the CC chemokines and the MCP-subfamily consisting of four members. CCL2 is chemotactic for monocytes and memory t-cells. It regulates the migration of the latter as well as natural killer cells. In human it is localized on chromosome 17 and is produced by a 99 amino acid residues precursor protein with a signal peptide that is cleaved off to produce the mature protein composed of 76 amino acids.[38,39] CCL2 has a size of 13 kDa.

Two regions in the primary structure are responsible for biological activity. Mutations in the first region from Thr-10 to Tyr-13 result in a decreased activity as well as mutations in the second region by introducing a proline between Ser-34 and Lys-35 or replacing those residues with Gly-Pro-His. Structural analysis disclosed a secondary structure consisting of four β-sheets and two helical regions. [38]

It has been shown, that GAG binding is essential for MCP-1 to exert cell migration *in vivo.* MCP-1 dimerizes in solution but the presence of GAGs induces tetramer formation. Although many chemokines form dimers, oligomerization is not necessary for receptor binding and chemotaxis in vitro but it is required for cell recruitment *in vivo*. [4]

MCP-1 is produced either constitutively or after induction by oxidative stress, growth factors, cytokines and exposure to inflammatory stimulation by different activated cell types. Although monocytes and macrophages are the main source for MCP-1, it is produced by a variety of cells including endothelial, fibroblasts, epithelial, smooth muscle and microglial cells. On monocytes, MCP-1 stimulates some activities including cytostaticity in human tumor lines, increase of cellular calcium levels and release of superoxide anions and lysosomal enzymes *in vitro*. Furthermore it has shown to chemoattract and induce histamine release by basophils and mast cells. [38,39] Unlike most other chemokines MCP-1 specifically recruits and activates CCR2-positive cells as it is binding exclusively to CCR2. Of this receptor there exist two spliced forms: CCR2A and CCR2B that differ only in their C-terminal tail but might activate different signaling pathways and exert different actions. [2,38,40]

MCP-1 belongs to the best-studied chemokines and offers potential for therapeutic intervention as it is involved in a variety of diseases that affect monocyte-rich cellular penetration, including multiple sclerosis, atherosclerosis, insulin-resistant diabetes and rheumatoid arthritis. [2,38] Due to high CCR2 expression resulting in MCP-1 recruitment, memory $CD4^+$ T cells and monocytes are primary targets for HIV-1 infection. MCP-1 plays also a role in cardiovascular diseases and cancer as the expression of MCP-1 in tumor cells is significantly correlated with a decrease of tumor.-associated-macrophage infiltration. [38] Different attempts have been made to interfere with CCL2 activity along with neutralizing monoclonal antibodies and drugs that block several

up-regulated chemokine receptors might be efficient if they are located upstream of the CCR2 expression. [2,38]Another approach is to modulate protein function *in vivo* and *in vitro* by increasing the affinity of MCP-1 to its respective GAG ligand and deleting signaling through CCR2. [2]

1.1.6 SDF-1

Stromal cell-derived factor-1/CXCL12 is a homeostatic chemokine that is highly conserved in mammalian species and constitutively expressed within tissues during organogenesis and adult life. Despite not containing an ELR-motif, it is angiogenic and affects pathophysiological cell motility associated with tumor metastasis.[6,41,42] There exist three isoforms arising from splicing a single gene, SDF-1α, SDF-1β and SDF-1γ.[41] SDF-1α is located on chromosome 10 compared to all other chemokines, which are either located on chromosome 4 or 17.[43] SDF-1α adopts the typical CXC chemokine structure, consisting of a three-stranded, antiparallel β-sheet followed by a C-terminal α-helix but the packaging is remarkably different compared to other chemokines. [43,44] It has an additional single turn of a 3^{10} helix that precedes the first β-sheet, shown in Figure 4. Even at high concentration SDF-1α remains monomeric. It has a distinct structure compared to other chemokines, which might occur due to sequential differences. [43]

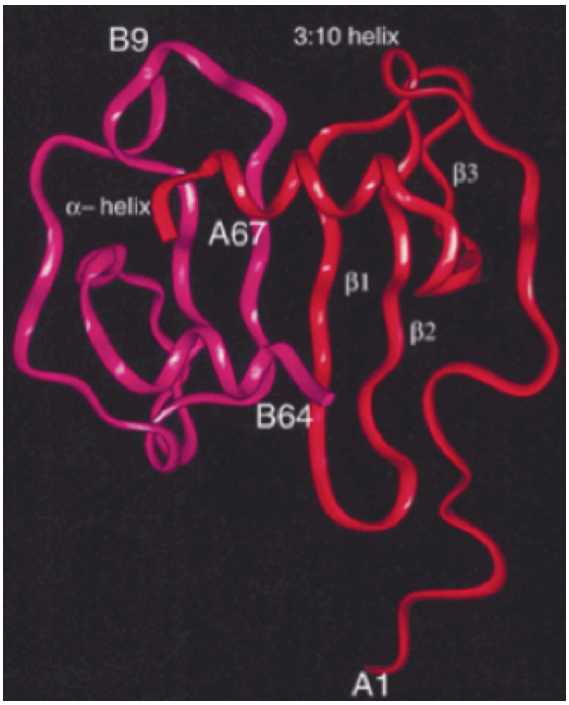


Figure 4 Structure of SDF-1. The extended N-terminal loop proceeding in the 3^{10} helix and the three antiparallel β-sheets followed by the C-terminal helix. Chris Dealwis et al. Immunology, Vol. 95, (1998) pp. 6941–6946

In crystalline form it is a dimer, but structure determination using NMR revealed a monomeric structure,[20] information about forming heterodimers with other chemokines is known with MIG and NAP-2. [45] SDF-1 is chemotactic for T-cells, monocytes, pre-B-cells, dentritic cells and hematopoietic progenitor cells. It is expressed not only in immune and hematic cells but also in neural, astroglial, microglial end endothelial cells. [46] SDF-1 plays an important role in tumor vascularization and regulation of processes involved in metastasis as chemoattraction and adhesion of malignant cells. [47]

The biological activity is mainly mediated by interaction with the specific receptor CXCR4 but also CXCR7.[6,41] As there is no other chemokine known to compete with SDF-1α for CXCR4 binding, SDF-1α is assumed to be unrivaled among α- and β-chemokines.[43] A RFFESH motif as well as the N-terminal domain, especially the first residue, is critical for specific interaction with CXCR4.[20,42–44] By this interaction, distinct pathways can be activated and furthermore involvement in cell proliferation, apoptosis and tumor progression is presumed. [48] The secretion of SDF-1 is also associated with tissue damage. Therapeutic intervention strategies are aimed to block the SDF-1-CXCR4 axis in order to inhibit metastasis. [47]

1.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are present on all animal surface cells and the extracellular matrix. With the exception of hyaluronic acid (HA) they are covalently linked to specific core proteins, thus forming the proteoglycan family. [49,50] The biosynthesis of proteo- glycans comprises the assembly of core proteins in the endoplasmatic reticulum, then the saccharides are adhered subsequently by glycosyl transferases in the Golgi. [21]

GAGs are linear, heterogeneous polysaccharide chains, assembled of repeating disaccharide units composed of N-acetyl-hexosamine (D-glucosamine or D- galactosamine) and an uronic acid (D-glucoronic acid or L-iduronic acid) or galactose. Either or both can be sulfated at different positions. GAGs vary in the composition of the saccharides, the linkage between the monosaccharides that can be either α- or β- configuration as well as in acetylation and N- and O-sulphation. Based on sulphation patterns, the main classes of GAGs can be divided into two groups: sulfated ones like chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin, and heparan sulfate (HS) and non-sulfated ones as HA.[49–51]

Moreover GAGs can be soluble, surface bound or constricted as soluble ectodomains. Extensive sulphation of GAGs results in a strong hydrophilic nature, their molecular domain encloses a large number of water molecules.[49]

Heparan sulfate is the most abundant GAG with a variable structure, mainly as a result of variations in the sulphation patterns. It is expressed on virtually all animal cells where it is initially produced cell-surface bound, but can also be shed in a soluble form with altered function.[21] Heparin, produced mainly by mast cells, is similar to HS but it contains more N-acetyl groups and fewer N- and O- sulfated groups. HS and heparin are the best studied of all GAGs whereupon heparin is often used as an analogue of HS as it is cheaper and easier to obtain.[21,51]

Through the regulation of their numerous protein partners, GAGs play an important role in various biological and pathophysiological processes including cell-cell communication, cell development and signaling as well as proliferation and differentiation of cells and inflammation

or angiogenesis.[51,52] Especially heparan sulfate as a major component of the glycocalix has been found to be involved in the regulation of leucocyte recruitment from the vessels to the site of tissue damage.[33] Also specifically denoting for heparin and HS is the interaction with functional proteins, which is often crucial for protein function.

As these physiological processes are induced by the interaction of GAGs with the respective proteins, proteoglycans also act as co-receptors by mediating protein function. [49,51] Due to conformational flexibility, sulphation patterns and the effect of pH to binding, the interaction of proteins with GAGs is of high complexity.[52] Considering the importance of GAG-protein interactions, interfering this process is an important target for drug design which is because of the complexity a challenging but promising task.[49,51,53] Hundreds of GAG binding proteins have been identified and their number is still growing. Nevertheless the number of well-characterized GAG-protein complexes is still small, among others because of the limited accessibility of human GAGs for analyzing purposes.[51] Depending on the number of sulfate and carboxylate groups on the chain, GAGs are charged highly negative. Therefore many GAG binding proteins are basic in nature and the main occurring binding is electrostatic.[21]
A number of proteins require selective GAG binding to exert their biological function.[53] In this regard, GAGs interacting with chemokines play an important role as this proteins are crucial in mediating cell migrational processes.[33]

1.3 Chemokine – Glycosaminoglycan Interaction

Chemokines interact with both GPCRs and Glycosaminoglycans.[54] To facilitate the cell migrational nature of chemokines, interaction with cell surface GAGs is crucial. [5]
The acidic, highly negative charge of GAGs facilitates the electrostatic interaction with the basic, positive charged chemokines.
All chemokines posses a GAG-binding domain. [55] Upon binding the establishment of a tissue bound chemokine gradient to avoid diffusion from the production site and the presentation of chemokines to leucocytes in tissue is facilitated.[55,56] Binding to GAGs for example is crucial for the ability to promote cell migration for MCP-1. [4] The binding motif recognized by chemokines is produced by the sulphation pattern of GAGs. [55]
Protein-GAG interactions have a low affinity and the target sequence is either not known or can be estimated only from screening animal derived oligosaccharides.[33] Compared to receptor interaction, chemokine-GAG binding shows a higher specificity provided through distinctly different GAG-binding epitopes among different chemokines. On chemokines there have been

defined GAG-binding epitopes as XBBXBX and XBBBXXBX, where B represents a basic amino acid. [57]

As many chemokines oligomerize upon GAG-binding, the oligomerization state provides another level of specificity and diversifies the characteristics of binding epitopes; one chemokine can interact selectively with different GAGs [56,57]

Interaction of chemokines with GAGs might present the N-terminus of chemokines to inflammatory cell surfaces where a signal response is induced by GPCR to drive leucocyte trafficking. [54]

1.4 Targeting Chemokine – Glycosaminoglycan Interaction. The CellJammer® approach

Chemokine-GAG interactions are associated with different inflammatory diseases. Therefore understanding these interactions is of high importance.[58] Different strategies for interfering GAG-chemokine interaction for therapeutically purposes have been explored so far.[1] Studies with mutant chemokines that still bind to their receptors but do not induce signaling were performed.[58,59]

To apply chemokines in a therapeutically effective way, they should be able to displace GAG-pre-bound wild type proteins to mask the binding site without affecting cells. ProtAffin has developed CellJammer® technology to combine impaired receptor binding with an increased GAG-binding affinity of selected wild type chemokines using structural and ligand specific information contained in natural GAG binding proteins. If the G-protein coupled receptor (GPCR) activity is knocked out, the chemokine decoys mask the specific GAG-binding site without inducing cell migration into inflamed tissues.[1,53,59]

To generate decoys, GPCR interaction and following neutrophil activation was annulled. In IL-8 e.g. this is achieved by deleting the N-terminal ELR motif. Amino acids in the GAG-binding domain were replaced by basic amino acids to achieve a higher GAG binding affinity without affecting the specific sequence. Some of the IL-8 mutants, called PA401, have shown a 100fold increased GAG-binding affinity towards HS and a drastic reduction in receptor CXCR1/2 activation.

GAG-binding affinity is increased through replacement with basic amino acids, which boost electrostatic interdependency and so affinity. To preserve the chemokines specificity for its GAG co-receptor, side chain contacts are conserved during the engineering process while side chains responsible for receptor activation are deleted or replaced by alanine residues. Other chemokines generated using CellJammer® approach include MCP-1 (PA910) and SDF-1

(PA1011). All decoys are derived by a structure conserving approach to make sure other functions as oligomerization are not disturbed. [33,53,59,60]

2 Material and Methods

2.1 Gel Shift Assay

In order to determine interaction between two distinct chemokines, gel shift assay was performed. In this case, the assay was performed to analyze the interaction of wt IL-8 (5.5 mg/mL DS 1101) and met MCP-1 (2.6 mg/mL DS 1121) from *E.coli.* MCP-1 was interchanged against MCP-1 (2.6 mg/ml DS 1206) from *Pichia pastoris* because of the methylation pattern. The assay is based on the observation that chemokines interacting with each other show different migration characteristics compared to single migrating chemokines. The electrophoretic mobility changes upon interacting, the complex moves less far through the gel compared to non-interacting chemokines. By means of the propagation in the gel, oligomerization of chemokines can be analyzed.

Buffer and gel preparation

10 x PBS

Sodium chloride	80 g
Sodium phosphate dibasic	113 g
Sodium phosphate monobasic	2.65 g
Dissolved in deionized water	1 L

PBST

1 x PBS	1 L
Tween 20	500 µL
Dissolved in deionized water	1 L

10 x TRIS-Glycine running buffer

1.92 M Glycine	144.1 g
0.24 M Trizma	29 g
Filled up with deionized water	1 L

pH was adjusted to 7 with 37 % HCl

TRIS-Glycerol sample buffer

0.5 M Tris-HCl pH = 6.8	25 mL
Glycerol	20 mL
Filled up with deionized water	100 mL

4 x TRIS gel buffer

Trizma	1.5 g
Filled up with deionized water	50 mL

pH was adjusted to 7.1

Gel preparation

Gel preparation for six 8 % PAA gels

30 % polyacrylamide	10.74 mL
4 x TRIS buffer	10.02 mL
10 % APS	100.02 µL
TEMED	0.6 µL
Water	19.08 mL

Gel preparation for six 12 % PAA gels

30 % polyacrylamide	16.2 mL
4 x TRIS buffer	10.02 mL
10 % APS	100 µL
TEMED	20.0 µL
Water	16.4 mL

The gel solution was prepared in a 50 mL Erlenmyer bottle. First PAA, deionized water and 4x TRIS buffer were added. The solution was mixed on a magnetic stirrer while adding APS (ammonium persulfate, #105K0700 Sigma) and TEMED (N,N,N,N-tetramethylenediamine, # 036K0694 Sigma) to obtain proper mixing.

The glass plates (0,75 mm # 1653308 BioRad) were clamped into the casting stands. 2 % agarose (gen X D1) was prepared by measuring 2 g agarose and fill up to 100 mL with deionized water. The solution was then heated up in the microwave and used to seal the glass plates to avoid leakage of the gel solution before polymerization.

The gel solution was then poured in between the glass plates using Pasteur glass pipettes. Subsequently the 10-well combs (0,75 mm # 1653359 BioRad) were put into the solution and the gel was polymerized at RT for at least 1 h. To store the gels, they were wrapped into wet paper towels and stored at 4 °C.

Native gel electrophoresis with wt IL-8 and MCP-1

For sample preparation, the respective amount of chemokine sample was filled up with PBS (# 367P066 PromoCell) to the required volume and incubated for 30 min at either RT or 4 °C. Subsequently, sample buffer was added in the required volume and the samples were applied to the gel. The concentrations of the chemokines as well as the total volume were varied during the experiment in order to figure out proper running conditions. In the beginning, a concentration of 8 µg chemokine was chosen and compared to a lower concentration, the application scheme is depicted in Table 2.1-1. The samples were incubated either at 4 °C or RT for 30 minutes. Before application to the gel, the same volume of sample buffer, 12.5 µL, was added.

Table 2.1-1 Sample preparation and application scheme for native gel electrophoresis. An exemplary scheme that was used with distinct concentrations for all gels. Here, 3 µg and 8 µg of sample war prepared.

	3 µg sample				**1:1 ratio**	**1:3 ratio**	**3:1 ratio**
Preparation			**1**	**2**	**3**	**4**	**5**
wt IL-8 [µL]	0.6		1.6		1.6	1.6	4.8
Met MCP-1 [µL]		1.2		3.1	3.1	9.2	3.1
PBS [µL]	11.7	11	10.9	9.4	7.8	1.7	4.6
Total volume [µL]	12.5	12.5	12.5	12.5	12.5	12.5	12.5

To further improve the assay, different conditions as runtime, pH and temperature of the buffer as well as pre-running the gel to avoid irregular warming of the gel were tested. To obtain smaller total volumes, wt IL-8 was diluted 1:10 with PBS to a concentration of 0,5 mg/ml. The samples were incubated at RT for 90 min, pH of the running buffer stored at RT, was adjusted to 7.05 and the gel was pre-run for 1 h. The application scheme and the sample preparation are shown in Table 2.1-2. A total amount of 3 µg sample was used, the empty slots were filled with sample buffer.

Table 2.1-2 Sample preparation and application scheme for a 12 % PAA gel, pre-run for 1h at 200 V and a runtime of 70 minutes at 200 V. A total amount of 3 µg sample was applied.

			1:1 ratio	**1:3 ratio**	**3:1 ratio**
Preparation	**1**	**2**	**3**	**4**	**5**
wt IL-8 [µL]	6.0		3.0	1.5	4.5
Met MCP-1 [µL]		1.15	0.6	0.9	0.3
PBS [µL]	4.0	8.8	6.4	7.6	5.2
Total volume [µL]			10		
Sample buffer [µL]			10		

To exclude problems with the resolution, a further decrease of sample concentration was tried. The pH was kept at around 7 to make migration in the gel possible, a higher pH might led to a decreased migration due to the high PI of the chemokines.

Table 2.1-3 Sample preparation and application scheme for a 12 % PAA gel, pre-run for 1h at 200 V and a runtime of 90 minutes at 200 V. A total amount of 2 µg sample was applied.

			1:1 ratio	1:3 ratio	3:1 ratio
Preparation	**1**	**2**	**3**	**4**	**5**
wt IL-8 [µL]	4.0		3.0	1.3	3.8
Met MCP-1 [µL]		0.8	0.6	0.7	0.2
PBS [µL]	6.0	9.2	6.4	8.0	6.0
Total volume [µL]			10		
Sample buffer [µL]			10		

For electrophoresis, the running buffer was diluted 1:10 with deionized water in a total volume of 700 mL for two gel chambers and adjusted to the desired pH using HCl and NaOH. Anode and Cathode were inverted to avoid the samples migrating out of the gel due the highly positive charge. The whole device was kept on ice during electrophoresis to avoid heating up, buffers were either stored at 4°C or RT before use. Voltage and runtime were changed during the experiment to figure out the right parameters.

For staining the gel, Coomassie Brilliant Blue was used. The gel was transferred into a staining tray, rinsed with deionized water and stained in 50 mL Coomassie staining solution for at least 30 minutes on the 3D orbital shaker. Afterwards the gel was rinsed again with deionized water and destained in 50 mL destaining solution for approximately 1h. Change of destaining solution was performed twice until the background becomes clear. The gel was then imaged using ChemiDoc XRS Imager and Image Lab software with the setting Coomassie.

2.2 Western Blot

Western Blot is another technique using native gel electrophoresis to determine interaction between chemokines. In this case, interaction is analyzed upon binding to specific antibodies, which are subsequently detected. Here Western Blot was performed to find out if interaction studies using this approach are feasible.

Buffer preparation

Blotting buffer for Western Blot

Trizma	7.28 g
Glycine	3.66 g
Filled up with deionized water	1 L

5 % dry milk for Western Blot

Dry milk powder	5 g
PBST	100 mL

0.1 % Ponceau S staining solution

Acetic Acid 100 %	25 mL
Ponceau S	500 mg
deionized water	475 mL

Gel electrophoresis for Western Blot

12 % polyacrylamid self-cast gels were used, one gel per electrophoresis chamber (Mini-PROTEAN® Tetra Cell,BioRad 165-8000). The sample preparation was performed according to the procedure for gel shift assay. Electrophoresis was run for 1.5 h at 200V using a Power supplier 164-5070 from BioRad. The Tris-Glycine electrophoresis buffer was adjusted to pH=7.01.

While running electrophoresis, the PVDF membrane (# K2JA2332H , Cat.-# ISEQ00010, Millipore) and blotting papers (# 170-3967, BioRad) were cut adapted to the size of the gel. Before finishing electrophoresis, the membrane was placed in 20 mL methanol for 3 seconds to wet it. Subsequently it was placed in 50 mL deionized water for 2 minutes. After gel electrophoresis was finished, the gel was rinsed with deionized Water and equilibrated with the membrane for 5 minutes in blotting buffer while shaking at the 3D orbital shaker (Edmund Bühler TL10).

The blotting sponges were wetted with blotting buffer and a sandwich prepared by placing a

sponge onto the anode-side of the blotting equipment (Semi-dry transfer system, BioRad) and put the gel onto the sponge. Above the gel, the membrane and the second sponge were placed. Air bubbles between the gel and the membrane should be avoided by scratching them out with a Pasteur pipette. Chemokines are plotted the other way round compared to other proteins. Gels were blotted for 30 minutes and for 1 h at 25 V as proper conditions had to be figured out first.

Subsequently, the gel was stained in Ponceau S 0.1 % for 1 minute and destained with deionized water. This was necessary to figure out whether gel electrophoresis worked, as the electrodes are inverted it is not possible to add a marker to make the gel bands visible during the run. The membrane was imaged using ChemiDoc XRS (BioRad) with silverstain setting.

For blocking, the membrane was incubated for 1 h in 50 mL 5 % non-fat dry milk in PBST while shaking at 22 rpm at the orbital shaker.

The primary antibody solution was prepared by measuring 10 mL 5 % non-fat dry milk in PBST into a 14 mL Nunc tube (Greiner) and adding 50 µL α-IL-8 rabbit polyclonal antibody (#F2111 Santa Cruz 200 µg/mL) to obtain a dilution of 1:200 and mixed gently.

The dry milk was discarded after blocking and the membrane was incubated with the primary antibody solution for 1 h while shaking at 22 rpm at orbital shaker at RT. Afterwards, primary antibody solution was discarded and the membrane was rinsed with PBST before three washing steps with 50 mL PBST, each for 10 minutes while shaking.

Meanwhile, the secondary antibody solution was prepared by measuring 10 mL 5 % non-fat dry milk in PBST into a 14 mL Nunc tube and adding 1 µL of peroxidase conjugated goat α-rabbit IgG-HRP (# 86831 , Jackson Immuno Research Labs) to obtain a dilution of 1:10.000.

PBST was discarded and the membrane incubated while shaking for 1 h with the secondary antibody at RT. Subsequently, the membrane was rinsed with PBST before three washing steps with 50 mL PBST, each for 10 minutes while shaking.

For detection, 600 µL of ImmunStar reagents A and B (# 170-5070 BioRad) were mixed in a 2 mL Eppendorf tube and pipetted onto the membrane placed in a sheet protector. The membrane was incubated for 1 minute at RT. Imaging was performed using ChemiDoc XRS Image Lab software with Western Blot setting.

2.3 FITC labeling

To perform isothermal fluorescence titration, the chemokines were labeled using fluorescein isothiocyanate (Sigma-Aldrich, Prod. # FITC1), a commonly used, highly sensitive fluorescent probe with an absorption maximum at 495 nm.
1 mg of met MCP-1 and wt MCP-1 and 500 µg of PA401, wt IL-8, PA910, PA1011, SDF-1 and HSA PA910 were labeled with a 4 molar excess of FITC.
For labeling, the respective amount of protein was used in a total volume of 500 µL filled up with PBS without Ca^{++} /Mg^{++} (PromoCell, Prod.# C-40232). The required volume of protein in PBS was calculated in Table 2.3-1 and filled up with the respective volume of PBS to obtain a final volume of 500 µL.

Table 2.3-1 Calculation of the required volume of protein to obtain a final volume of 500 µL of chemokine in PBS. HAS PA910 was diluted 1:5 because it was high concentrated. To PA1011 no PBS was added, as the concentration was low.

Chemokine	MW	c [mg/mL]	µL protein	µL PBS
wt MCP-1	8699	2.6	385	185
Met MCP-1	8730	3.4	294	206
PA401	7676	5.6	89.3	410.7
PA910	8820	2.9	172.4	327.6
wt IL-8	8386	5	100	400
PA1011	7248.7	0.91	549.5	
HSA PA910	74980	7.57	66.05	433.95
SDF-1	8094.6	5.55	90.1	409.9

The required amount of FITC was calculated for all chemokines, using formula (1)

$$\frac{mg\ Protein}{MW\ Protein} * factor\ of\ molar\ excess * MW\ FITC = mg\ FITC \qquad (1)$$

10 mg of FITC were dissolved in 1 mL DMSO in an eppendorf tube to obtain a concentration of 10 mg/mL. The necessitated volume of FITC in Table 2.3-2 was calculated for each chemokine, using formula (2) and the respective volume was added to each protein in PBS solution.

$$\frac{1000\ \mu l}{10\ mg} * mg\ FITC = \mu l\ FITC \quad \textbf{(2)}$$

Table 2.3-2 Calculation of the required volume of 10 mg/mL FITC for each chemokine.

Chemokine	FITC [mg]	FITC [µL]
wt MCP-1	0.18	18
Met MCP-1	0.18	18
PA401	0.10	10.14
PA910	0.09	8.83
wt IL-8	0.09	9.28
PA1011	0.11	10.74
HSA PA910	0.01	1.04
SDF-1	0.10	9.62

Upon addition of the calculated volume of FITC in Table 2.3-2 the FITC-chemokine solutions were incubated at RT while shaking for 2 h. After incubation, the samples were desalted using Zeba Spin Desalting Columns from (Thermo Scientific, Prod.# 89889) according the instructions. The columns were placed in 15 mL Nunc collection tubes (Thermo Scientific, Prod.#339650) with the cap loosen and centrifuged for 2 minutes at 1000 x g in the Sigma laboratory centrifuges 6K 15 to remove the storage solution. A mark was placed at the side where the compact resin remained to replace the columns in each centrifugation step in the same position. Subsequently, 1 mL PBS without Ca^{++} /Mg^{++} was added to the columns and the centrifugation step was repeated. This was recapped three times discarding the flow through. For sample loading, the columns were placed into new collection tubes and the samples were loaded. The samples were centrifuged at 1000 x g for 2 minutes and were stored in black 1,5 mL microcentrifuge tubes.

The concentration of labeled chemokines was determined using Jasco V530 Photometer with a TrayCell (Hellma) at a wavelength of 280 nm. The labeling ratio and yield was calculated with the absorption measured at a wavelength of 495 nm. Upon calculation of the concentration in Table 2.3-3, isothermal fluorescence titration was performed with the labeled chemokines.

Table 2.3-3 Concentration determination of FITC labeled chemokines as well as calculation of yield and labeling degree.

Chemokine	c [mg/mL]	Degree of labeling [moles FITC/mole protein]	Yield [%]
FITC wt MCP-1	1.35	0.007	51.9
FITC Met MCP-1	2.27	0.005	66.8
FITC PA401	0.91	0.009	16.2
FITC PA910	1.95	0.006	67.1
FITC wt IL-8	1.51	0.007	30.1
FITC PA1011	2.09	0.004	230
FITC HSA PA910	0.51	0.015	6.79
FITC SDF-1	0.54	0.014	9.81

2.4 Isothermal Fluorescence Titration

To determine binding affinities of chemokines among each other, IFT was applied. Upon binding, the fluorescence gets quenched or de-quenched depending on the chemokine and K_D values can be calculated. The fluorescence originates from the FITC label at a wavelength of 495 nm or in case of titration with glycosaminoglycans at a wavelength of 280 nm from an internal tryptophan of the chemokine.

2.4.1 IFT with FITC labeled chemokines

FITC labeled chemokines were titrated against each other to analyze possible interaction.
The spectrofluorometer Jasco FP 6500 was switched on half an hour before measurement and the waterbath (Lauda Ecoline Staredition RE204) temperature was held at 20 °C. The cuvettes were stored in nitric acid and cleaned before use under running water. Afterwards the cuvettes were rinsed with deionised water and dried with compressed air.

Sample preparation

500 µL of PBS without Ca^{++} /Mg^{++} (PromoCell, Prod.# C-40232) were pipetted into the cuvette and the respective calculated volume for the chemokine solution was removed and replaced by the volume of chemokine in Table 2.4-1 of the chemokine solution to achieve a final concentration of 700 nM. Including the molecular weight and the previous determined concentration of the chemokine the volume was assessed.

Table 2.4-1 Preparation of a 700 nM chemokine solution

Chemokine	MW	c [mg/mL]	µg/500 µL	µL Chemokine
FITC wt MCP-1	8699	1.35	3.04	2.26
FITC Met MCP-1	8730	2.27	3.06	1.34
FITC PA401	7676	0.91	2.69	2.96
FITC PA910	8820	1.95	3.09	1.59
FITC wt IL-8	8386	1.51	2.94	1.95
FITC PA1011	7248.7	2.09	2.54	1.21
FITC HSA PA910	74980	0.51	26.2	51.1
FITC SDF-1	8094.6	0.54	2.83	5.21

The titrant chemokine was prepared in a concentration of 500 nM. Depending on the chemokine, in some cases the concentration had to be increased to achieve saturation. After the 700 nM chemokine solution was prepared and mixed gently with the pipette, it was equilibrated for 30 minutes. Then the first spectrum was recorded that corresponds to the titrant free sample. The emission upon excitation at 495 nm was recorded over a range from 500 nm to 600 nm. After recording, titrant chemokine was added and mixed carefully. After exactly 1 minute of equilibration, the emission was recorded which corresponds to the respective chemokine in presence of the chemokine titrant con- centration. The addition of titrant chemokine was repeated several times, increasing concentration until saturation was achieved. This was depended on the chemokine and the used titrant. The measurement was performed in a threefold determination. For analysis, every recorded spectrum was corrected from background signal. Therefore the background was detected by preparing 500 µL of PBS but without adding a protein. Then it was titrated with the respective titrant chemokine in the same increasing concentration steps as the chemokine solutions. To correct the spectra from background

emission, the background signal was subtracted from the recorded spectrum. The normalized change in fluorescence was calculated using formula (3).

$$\frac{\Delta F}{F_0} = \left|\frac{Area_i - Area_0}{Area_0}\right| \quad (3)$$

Where ΔF is the normalized change in fluorescence, F_0 the initial area before adding the titrant, $Area_0$ the area recorded in absence of a ligand and $Area_i$ represents the spectra obtained after addition of a ligand. The leading sign depends on whether fluorescence is quenched or de-quenched.

The final analysis was performed using origin 8.1 G software to plot $\pm\frac{\Delta F}{F_0}$ versus the increasing concentration of the titrant and to calculate dissociation constant of the interaction K_D. The latter was calculated by applying Formula (4).

$$F = F_i + F_{max}\frac{K_D + [P]\,[L] - \sqrt{(K_D + [P] + [L])^2 - 4\,[P][L]}}{2\,[P]} \quad (4)$$

F_i and F_{max} are the initial and the maximum fluorescence, [P] and [L] are the total concentrations of chemokine and ligand.

2.4.2 IFT with FITC labeled chemokines after incubation with heparin

As some chemokines oligomerize in presence of GAGs, different chemokines that show no interaction in the previous experiment as well as those which did interact were titrated against each other upon incubation with heparin to figure out whether binding to heparin changes the interaction profile.

Sample preparation

A 700 nM solution of the FITC labeled analyte chemokine is prepared as in the previous experiment but this time 1 µM of Heparin (Iduron, BN5) was added additionally. The starting volume was 500 µL. As a titrant, a solution with a concentration of 500 nM of the respective non-labeled chemokine is prepared in PBS as described before. Upon an equilibration time of 30 min at RT, titration was performed in increasing ligand concentrations. The chemokines used for titrations are depicted in Table 2.4-2.

Table 2.4-2 Titration of FITC labeled chemokines equilibrated with 1 μM heparin.

FITC PA910 + Heparin	PA401
FITC IL-8 + Heparin	PA401
FITC SDF-1 + Heparin	PA401
FITC PA910 + Heparin	PA1011
FITC IL-8 + Heparin	PA1011
FITC wt MCP-1 + Heparin	PA1011
FITC wt MCP-1 + Heparin	PA401
FITC PA910 + Heparin	Met SDF-1
FITC PA1011 + Heparin	PA401
FITC IL-8 + Heparin	wt MCP-1
FITC PA1011 + Heparin	wt MCP-1
FITC wt MCP-1 + Heparin	Met SDF-1
FITC IL-8 + Heparin	Met SDF-1
FITC PA401 + Heparin	Met SDF-1
FITC Met SDF-1 + Heparin	Met SDF-1
FITC PA401 + Heparin	PA910
FITC PA910 + Heparin	PA910
FITC Met SDF-1 + Heparin	PA910
FITC PA1011 + Heparin	PA910
FITC PA401 + Heparin	PA1011
FITC PA1011 + Heparin	wt IL-8
FITC Met SDF-1 + Heparin	wt IL-8

The settings were adjusted to the respective chemokine. The fluorescence was measured at a wavelength of 495 nm. Analysis was performed using Origin 8.1 G software.

2.5 Surface Plasmon Resonance

To investigate the binding affinity of heparin sulfate to the FITC labeled chemokines in comparison to not labeled chemokines by SPR a SA-chip (carboxymethylated dextran pre-immobilized with streptavidin) coated with heparan sulfate (Iduron BN 5, CH, Batch 74) was used.

The analysis was performed using a Biacore X100 model. In one run, two chemokines were analyzed: a FITC labeled one and the respective non-labeled one. For sample preparation, a dilution row was prepared by starting with 500 µL PBST and adding the calculated volume of each chemokine depicted in Table 2.5-1.

Table 2.5-1 Surface Plasmon Resonance to determine the different binding affinities of FITC labeled mutant and wild type chemokines to non-labeled chemokines towards heparan sulfate.

FITC PA401

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
7676	*1*
0,91	*118,551329*

fuer 0,8 uM in 500 ul PBST

3,37

PA401 TOX Batch

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
7676	*1*
5,6	*729,546639*

fuer 0,8 uM in 500 ul PBST

0,55

FITC wT IL-8

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8386	*1*
1,51	*180,062008*

fuer 10 uM in 500 ul PBST

27,77

wT Il-8

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8386	*1*
5	*596,231815*

fuer 10 uM in 500 ul PBST

8,39

FITC PA1011

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
7248,7	*1*
2,09	*288,327562*

fuer 0,8 uM in 500 ul PBST
1,39

PA1011

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
7248,7	*1*
0,91	*125,539752*

fuer 0,8 uM in 500 ul PBST
3,19

FITC met SDF-1

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8094,6	*1*
0,544	*67,2052974*

fuer 10 uM in 500 ul PBST
74,40

met SDF-1

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8094,6	*1*
5,55	*685,64228*

fuer 10 uM in 500 ul PBST
7,29

FITC PA910

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
8820	*1*
1,95	*221,088435*

fuer 0,8 uM in 500 ul PBST
1,81

PA910

nM	Serial dilution	PBST (ul)
0	200	
25	200	200
50	200	200
100	200	200
200	200	200
400	200	200

mg/ml	*M*
8820	*1*
2,9	*328,798186*

fuer 0,8 uM in 500 ul PBST
1,22

FITC wT MCP-1

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8699	*1*
1,35	*155,190252*

fuer 10 uM in 500 ul PBST
32,22

wT MCP-1

nM	Serial dilution	PBST (ul)
0	200	
250	200	200
500	200	200
1000	200	200
2500	200	200
5000	200	200

mg/ml	*M*
8699	*1*
2,6	*298,884929*

fuer 10 uM in 500 ul PBST
16,73

For mutant chemokines, the initial concentration in the first tube is 800 µM, for the respective wild type it is 10 µM. Biotinylated heparan sulfate was injected into the flow cell of a SA chip that

is coated with streptavidin .The flow cell was conditioned with wash buffer for Biacore 1 M NaCl in 50 mM NaOH, sterile filtered (13.4.12 MN). To determine interaction, the chemokines are dissolved in sample buffer PBST and injected with a continuous flow into the flow cells onto immobilized heparin sulfate. The equilibrium dissociation constant K_D was determined.

3 Results and Discussion

3.1 Gel shift assay

Gel shift assay was performed with different approaches to determine the ideal runtime and composition of samples. Due to some difficulties referred to resolution and migrational characteristics, in order to improve this assay it was performed with only two chemokines MCP-1 and IL-8.

At the beginning 8 % PAA gels were tested with a runtime of 5 to 6 hours with 100 V. Due to high temperature evolving during electrophoresis there was no resolution and the bands were fuzzy. In some cases, no bands were visible as the samples probably moved out of the gel due to long runtime and low PAA percentage. Therefore the percentage of PAA was increased to 12 % to make the gels more stable and heat resistant as well as to avoid the proteins migrating out if the gel by increasing the degree of cross-linking. . This has shown better results in direct comparison.

As shown in Figure 3.1-1, using a 12 % PAA gel and voltage of 100, no resolution was obtained and the chemokine migration was low despite a runtime of 6 hours. With a view to Figure 3.1-1 it can be seen that no estimation about potential interaction is viable. The samples run approximately to the middle of the gel and then stopped. Apart from that, the bands were uneven and not clear. To improve the migration of the proteins, an increase of voltage in small steps was tested to facilitate the migration of chemokines into the gel, which did not change the result. Also the runtime was decreased and the voltage was increased.

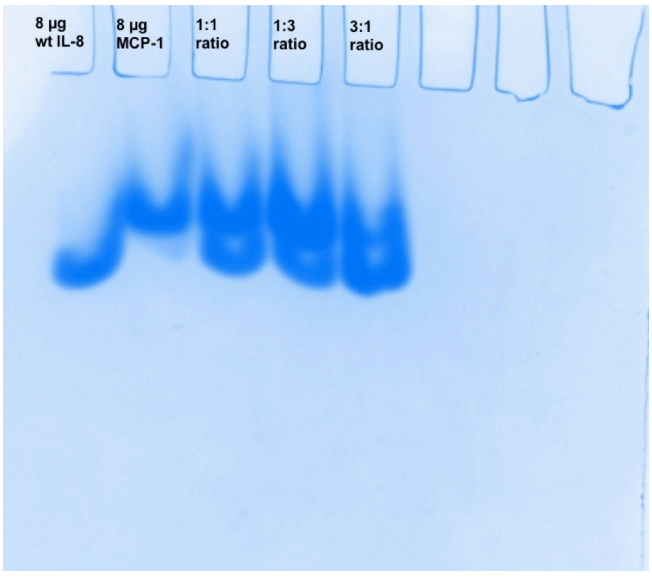


Figure 3.1-1 12 % PAA gel, runtime 6 h with 100 V. A total amount of 8 µg sample was used.

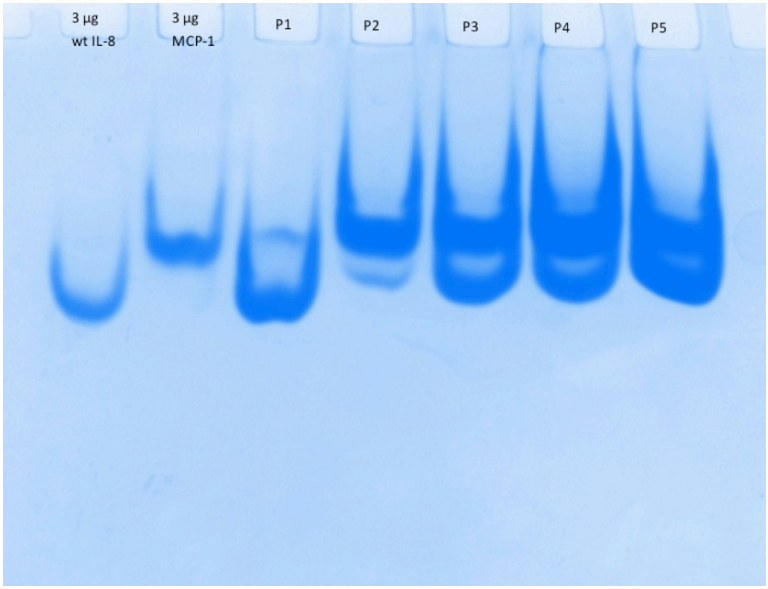


Figure 3.1-2 12% PAA gel, runtime 1h with 200 V. P1 to P5 depict the sample preparation shown in **Table 2.1-1**. In this gel different amounts of sample have been used. As can be seen in the figure, an amount of 3 µg leads to a better resolution compared to a total of 8 µg sample illustrated in different ratios as P1 to P5.

It has been shown that a decrease of sample amount leads to a higher resolution. The assay was started using 8 µg sample, also shown in Figure 3.1-1, which leads to a lower resolution compared to a lower amount of sample depicted in Figure 3.1-2. Using a higher amount of sample it was not possible to distinguish the bands and give information about a possible interaction.

Often occurring troubles were askew running bands. During electrophoresis the gels were heating up, especially in the middle, which explains why most bands run straight at the beginning and diffuse after a certain runtime. As the mobility of chemokines is also dependent from temperature, non-uniform heat distribution leads to aslant bands, as heat loss is lower in the middle of the gel. This problem was tried to circumvent by keeping the device on ice. In some cases it worked but the results were not reproducible. It seems as the runtime in combination with higher voltage might have been too long, but decreasing it, the samples migrated not far enough.

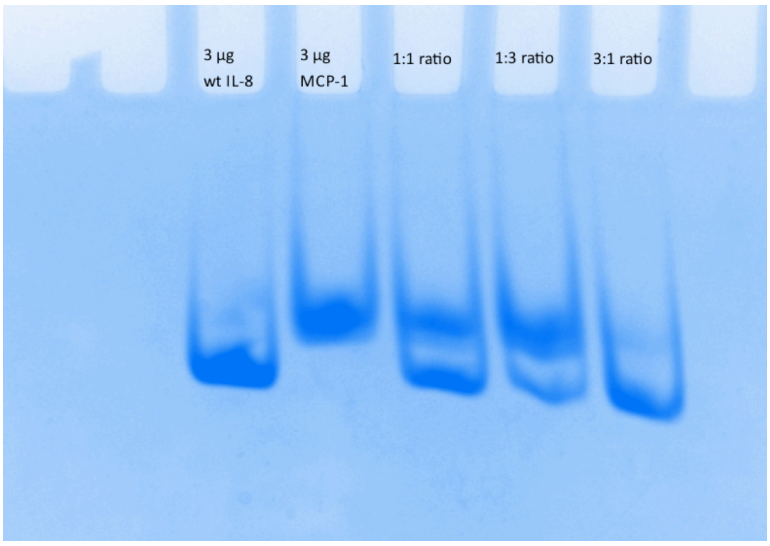


Figure 3.1-3 12% PAA gel, pre-run for 1h with 200 V, runtime 70 min with 200 V. Sample preparation is depicted in **Table 2.1-2**.

Another approach was to place the samples in different slots to figure out if the position on the gel might be responsible for migrational characteristics also did not

show a different result.
The next approach was to pre-run the gels to obtain a consistent warming up of the gels, avoid heating up in the middle of the gel and obtain a better separation of the proteins. In fact, as depicted in Figure 3.1-3 the result respective resolution was better but it did not solve the problem with the aslant bands. Storing the running buffer at RT before use also improved the results.
To further improve the assay, different runtimes were tested. Also the pH of the buffer was changed to obtain a better resolution but due to the high PI of the used basic chemokines the leeway was confined to a pH range around 7. At this pH range, chemokines are kept in their native form. Using Laemmli buffer with a pH of 8,5 showed no resolution at all only a single band smear. This lead to the assumption, that maybe pH and not voltage or runtime might be responsible for aslant running bands. The problem was that an increased pH is too closed to the PI of the chemokines so there was also no resolution obtained and therefore there was low possibility to change the pH. As the PI of the chemokine is higher than the pH of the buffer, cathode and anode had to be turned around. Because of this it was not possible to add a common marker to see how far the bands were running. The high PI of the chemokines might also cause trouble with migrating in the gel, which might explain the low migration observed in all gels. For a high PI, Native PAGE might not be the best technique as separation is performed only based on PI. Furthermore high content of salt might lead to migrational troubles that could occur by adjusting the pH using HCl, which was performed in this case. Due to a high ionic strength, the electrophoretic mobility decreases.
All in all despite different approaches and trying to adjust the parameters it was not possible to use Native PAGE as a reliable method for interaction studies. On some gels it seems that there occurs not interaction between MCP-1 and wt IL-8 but the bands were not clear enough to allow more than a guess. The smear visible in all gels could be oligomerization products, which furthermore hinders identification of interaction.

Still, the best results were obtained using running buffer stored at RT, pre-run the gel, using a runtime of approximately 1 hour and keeping the pH closed to 7 and a sample amount of 2 to 3 µg. Nevertheless the results were not reproducible and it was not possible to obtain consistent bands using Native PAGE. The problem is probably the high PI of the chemokines and also the unpredictable characteristics of native gels as in some cases bad polymerization occurred or the gel seemed to heat up in the middle although the devices were kept on ice all the time. As the results were not predictable at all, this method seemed not to be feasible to determine interaction of chemokines. It might allow guessing about interactions but as the chemokines

migrated slowly in the gel, mainly maximum to the middle of the gel, it does not provide secure information about interaction.

3.2 Western Blot

As parameters for chemokine western blot using Native PAGE were not known, they had to be figured out first. In Figure 3.2-1 and Figure 3.2-2 the native gels are shown that were blotted for different time to figure out the best blotting time.

The 60 min blotted gel had a high, background (Figure 3.2-1). A blotting time of 30 min, shown in Figure 3.2-2 was determined to work best.

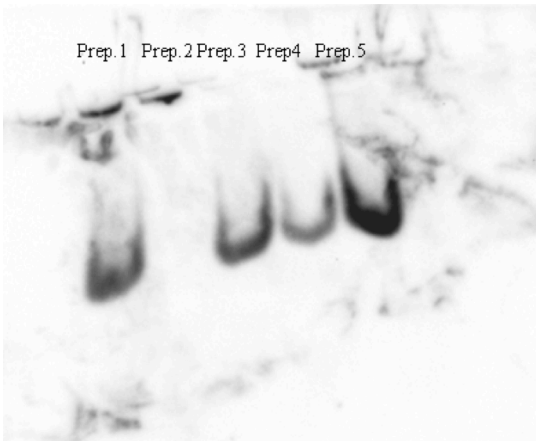


Figure 3.2-1 Determination of Western Blot parameters. Western Blot was performed using wt IL-8 antibody. The gel was blotted for 60 min.

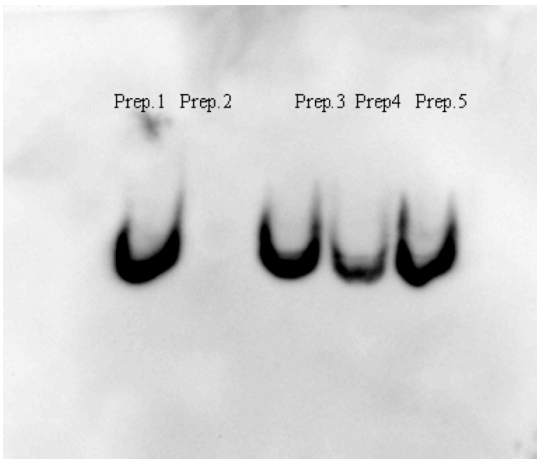


Figure 3.2-2 Determination of parameters. This gel was blotted for 30 min.

The problems occurring with Native PAGE continued with Western Blot. As the gels were not useable owing to low electrophoretic separation of the chemokines, also Western Blot was not feasible for interaction studies. This experiment was performed mainly to figure out if and how this analysis can be performed. Therefore only one chemokine was detected with the antibody, in Figure 3.2-1 MCP-1 and Figure 3.2-2 wt IL-8. Blotting the gel to the PVFD membrane worked; also the right blotting time was determined. It has been shown that chemokines are blotted the other way round, above the gel, due to their highly positive charge.
Western Blot was also performed for MCP-1 and repeated for wt IL-8 under the figured out conditions. As in the previous case, the pitfall was again Native PAGE.
To analyze potential interaction, electrophoresis has to be performed under native conditions but this seems to not be a feasible approach for Western Blot. In literature, Western Blots

performed with chemokines are run under denaturing conditions and with lower voltage, which did not work in this experiment. [61]

3.3 IFT

As shown in Table 2.3-3, the yield of FITC labeled chemokines is quite low referred to the initial concentration. To optimize this method there needs to be standardized a way to avoid losing protein during the desalting steps. In case of HSA PA910 and SDF-1, the labeling efficiency in contrast to the other chemokines is higher by a factor of 10 compared to the other chemokines, but in parallel, more protein precipitated. A high degree of labeling is useful in this case to increase sensitivity, as the assay applied is fluorescence based. As FITC can bind covalently only to uncharged, free primary amines at the amino terminus and on Lys residues of the protein[62], a low labeling efficiency might be explained by insufficient free amines available which seems unlikely in the case of the overall highly basic chemokines. The pH of the used PBS was 7,4 according literature, an increase of pH to 9 while labeling might lead to a better labeling efficiency[63]. Apart from a higher pH also an increase of temperature and a prolonged reaction time can accelerate labeling. [64]

3.3.1 IFT with FITC labeled chemokines

To quantitate the interactions between chemokines and their respective mutants among each other, IFT was used.
In most cases, the fluorescence was quenched but also sometimes de-quenched, leading to a change in the fluorescence $+\frac{\Delta F}{F0}$. An overview of the results is given in Table 3.3-1.

Table 3.3-1 An overview of the results is given. In some cases it seems the chemokines were interacting slightly which is marked with a "?". Interacting and non-interacting chemokines as well as quenched or de-quenched fluorescence are marked either with "+" or "-"

Protein	Ligand	Interaction	$\pm\frac{\Delta F}{Fo}$
FITC IL-8	PA401	-	-
FITC PA910	PA401	-	-
FITC PA1011	PA401	?	+
FITC PA401	PA401	?	+
FITC SDF-1	PA401	-	-
FITC wt MCP-1	PA401	-	-
FITC HASPA910	PA401	-	-
FITC PA401	HSA PA910	?	-
FITC IL-8	HSA PA910	-	+
FITC PA910	HSA PA910	-	-
FITC PA1011	**HSA PA910**	+	-
FITC SDF-1	HSA PA910	-	-
FITC wt MCP-1	**HSA PA910**	+	+
FITC IL-8	IL-8	-	-
FITC PA401	IL-8	?	-
FITC wt MCP-1	IL-8	?	-
FITC HSA PA910	IL-8	-	-
FITC PA910	IL-8	?	-
FITC PA1011	**IL-8**	+	-
FITC SDF-1	**IL-8**	+	-
FITC IL-8	**PA910**	+	-
FITC PA401	PA910	-	-
FITC PA910	PA910	-	-
FITC PA1011	**PA910**	+	-
FITC SDF-1	**PA910**	+	-

FITC wt MCP-1	**PA910**	**+**	**+**
FITC HASA PA910	PA1011	-	-
FITC IL-8	PA1011	-	-
FITC PA401	PA1011	-	-
FITC PA910	PA1011	-	+
FITC PA1011	**PA1011**	**+**	**-**
FITC SDF-1	PA1011	?	-
FITC wt MCP-1	PA1011	-	-
FITC IL-8	SDF-1	-	-
FITC PA401	SDF-1	-	-
FITC PA910	SDF-1	-	+
FITC PA1011	SDF-1	-	-
FITC SDF-1	SDF-1	-	-
FITC wt MCP-1	SDF-1	-	+
FITC IL-8	wt MCP-1	?	-
FITC PA401	**wt MCP-1**	**+**	**-**
FITC PA910	wt MCP-1	-	-
FITC PA1011	**wt MCP-1**	**+**	**-**
FITC SDF-1	**wt MCP-1**	**+**	**-**
FITC Met MCP-1	**Met MCP-1**	**+**	**+**
FITC wt MCP-1	**wt MCP-1**	**+**	**+**
FITC Met MCP-1	**wt MCP-1**	**+**	**+**
FITC wt MCP-1	**Met MCP-1**	**+**	**+**

PA401 showed to have no interactions when used as ligand in the experiment. Only slight interaction occurs for PA401 interacting with FITC PA401, shown in Figure 3.3-1 and PA1011. In the first case the slight interaction is not remarkable, as PA401 itself undergoes dimerization under specific conditions. It could be interesting to find out whether PA401 would show better interaction upon binding to heparin as this can induce dimerization in the wild type. Slight interaction in this case means that the change in fluorescence intensity was too small to significantly indicate binding.

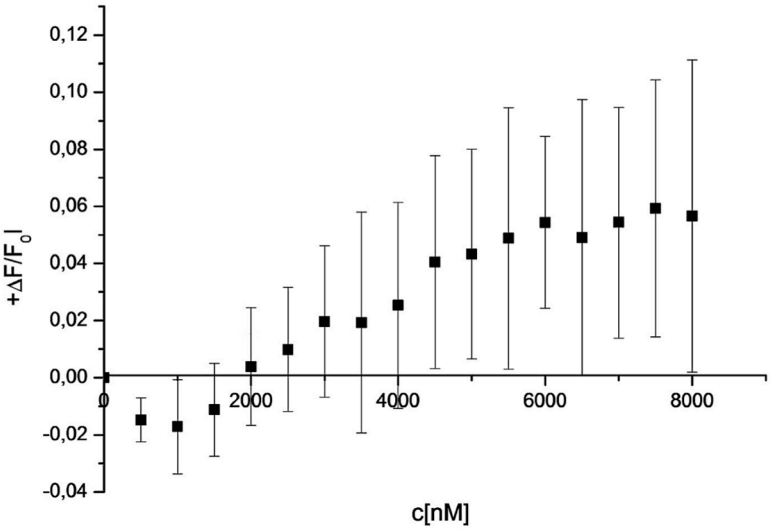


Figure 3.3-1 IFT FITC PA401 titrated with PA401.The given results do not sure indicate dimerization of PA401 but as shown a reaction occurs.

Also using FITC labeled PA401 did not show interaction with any of the utilized chemokines. Only the mutants' counterpart wt IL-8 interacted slightly in the same way as PA401 with FITC PA401. Also this result could have been expected as by generating the mutant form, only non-crucial amino acids are replaced, so the inter-molecular interaction characteristics with other chemokines should not

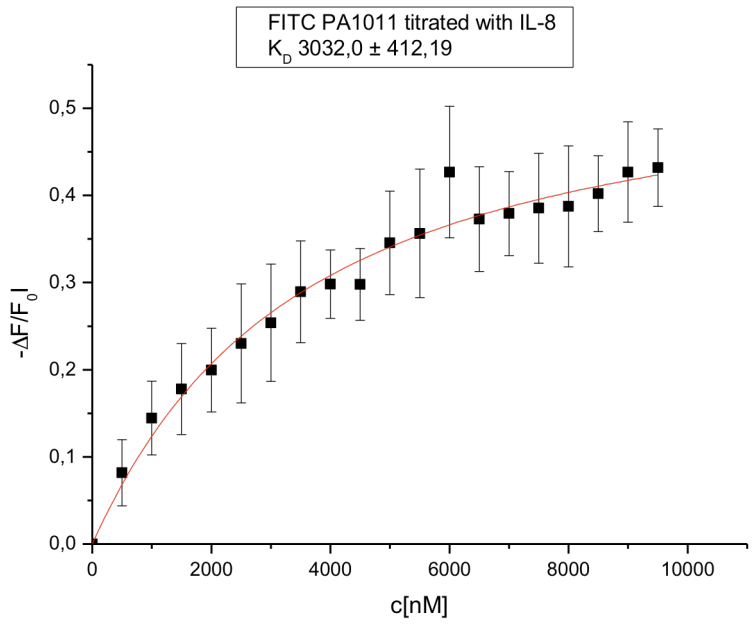


Figure 3.3-2 IFT FITC PA1011 with wt IL-8. The results clearly show this to chemokines forming heterooligomers.

have been affected.

Wt IL-8 was found to interact with FITC labeled PA1011 and SDF-1 (depicted in Figure 3.3-2 and Figure 3.3-3) and also slightly with wt MCP-1 and PA910. In comparison, defected from Table 3.3-1 it seems that wt Il-8 is more reactive with other chemokines compared to PA401. PA401 tends to be monomeric whereas IL-8 shifts between monomer and dimer. As IL-8 occurs in monomeric and dimeric forms naturally[65], it is interesting that in our experiment wt-IL 8 did not interact with itself. This might be attributed to the fact, that IL-8 is a monomer at nanomolar concentrations, which did not change significantly during the titrations. Binding to its receptor can induce dimerization that was not analyzed in this work.

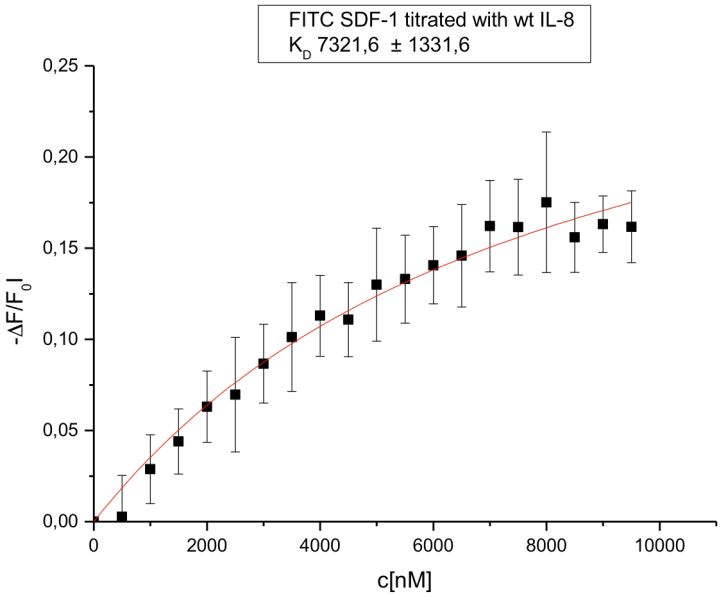


Figure 3.3-3 IFT FITC SDF-1 with wt IL-8. Also the non-mutant chemokine interacts with IL-8 but less distinctive.

Indeed it might be interesting, to figure out the impact of labeling, to analyze a possible interaction of wt IL-8 with FITC IL-8 following binding to heparin, as dimerization upon binding to heparin was demonstrated already[66]

PA910, the mutant isoform of MCP-1 interacted as ligand with FITC labeled wt IL-8, PA1011 and SDF-1.

Furthermore it interacted with wt MCP-1, interestingly however, no interaction with FITC PA910 was observed. In contrast to the not labeled PA910 deployed as ligand, FITC labeled PA910 interacted somewhat with wt IL-8 but not with the other chemokines. Human serum albumin coupled PA910 on the other hand showed different interaction pattern compared to PA910. It interacts also with PA1011 and slightly with PA401 and wt MCP-1. Compared to HSA PA910, PA910 interacted with FITC wt IL-8 and SDF-1. Therefore the HSA-fusion to extend the chemokines half-life time seems to interfere with the interaction of those chemokines. To confirm this result, i.e. if the HSA-fusion alters interaction with other

chemokines compared to PA910, further investigations are necessary.

FITC labeled HSA PA910 showed no interaction with PA401 and PA1011. As not enough sample of FITC labeled HSA PA910 was available, interaction studies with MCP-1 were not performed. All in all, FITC HSA PA910 does not interact with any other of the tested chemokines. Due to the low amount of FITC HSA PA910 available, it was not possible to investigate potential interaction with all used chemokines.

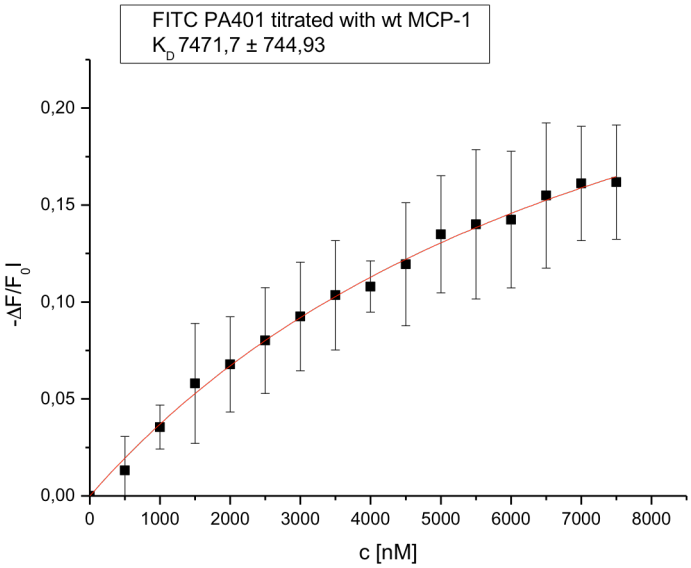


Figure 3.3-4 IFT FITC PA401 with wt MCP-1. Interaction occurs only one way.

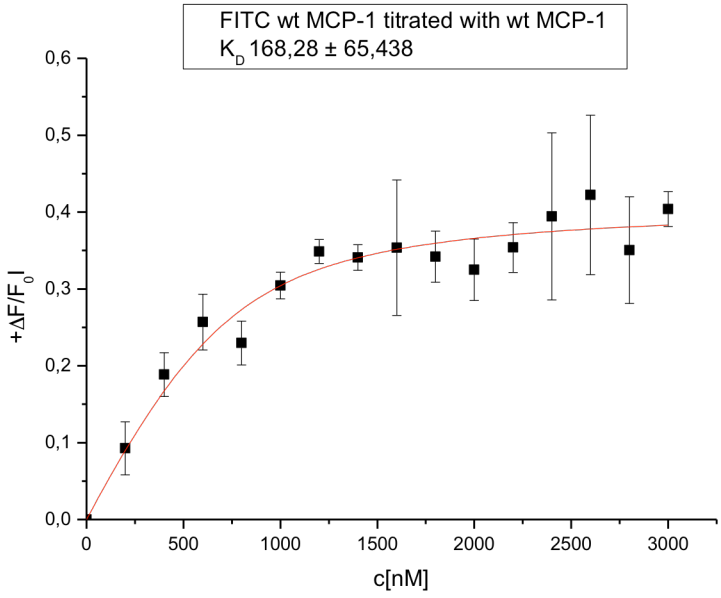


Figure 3.3-5 IFT FITC wt MCP-1 with wt MCP-1. As shown in the figure, MCP-1 forms oligomers with itself.

Wt MCP-1 exhibited a different interaction. It interacted with FITC wt IL-8, wt MCP-1, HSA PA910 and also SDF-1 and PA401 (Figure 3.2-2), which is not the case vice versa. The best interaction was obtained when binding to itself, as shown in Figure 3.3-5 or to Met MCP-1. The interaction with FITC PA401 is shown in Figure 3.3-4, where saturation was not achieved. Despite PA910 is interacting with FITC wt MCP-1, as shown in Figure 3.3-8, this is not the case when FITC PA910 was used. Although wt MCP-1 has a high binding affinity for itself, assessed by a K_D of 168 nM (Figure 3.3-5), the mutant variant does not oligomerize. Especially in this case it could be hypothesized that the mutation, although it only comprises the replacement of

non-crucial amino acids in the GAG-binding site might have some impact on the oligomerization pattern of the respective chemokine.

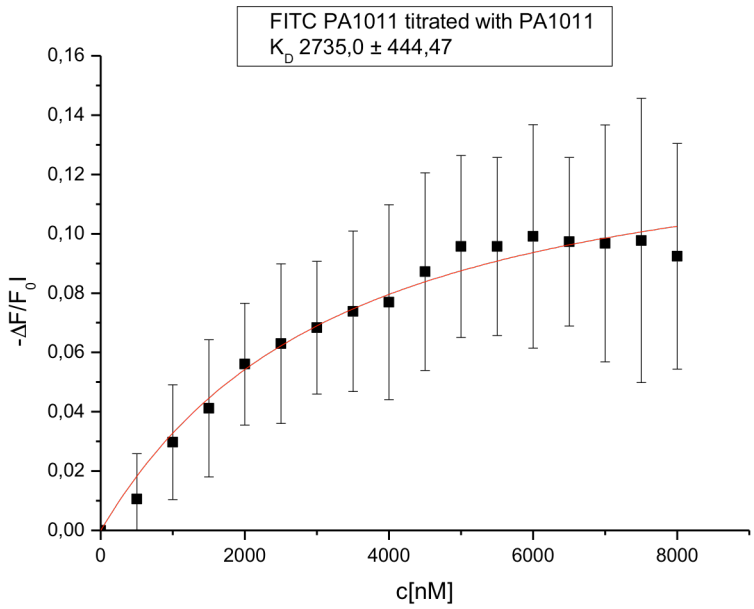


Figure 3.3-6 IFT FITC PA1011 with PA1011. The mutant but not the wild type was found to oligomerize in solution.

Nevertheless the situation is inverted relating to PA1011 and SDF-1. In this scenario, PA1011 has shown significant interaction with FITC PA1011, presented in Figure 3.3-6 whereas SDF-1 did not form oligomers with itself. This is an expected result as found in literature SDF-1 does not dimerize in solution without addition of a stabilizing counterion and only in non-acidic pH range.[67] Still the mutant and the wild type react in a completely different way. PA1011 as titrant interacts with itself and rudimentary with SDF-1. The other way round, interaction has shown to be much better Considering the published results it is surprising that PA1011 oligomerizes although no stabilizing counterions as heparin, or sulfate were added. As the used buffer contains phosphate, which also acts as a stabilizer for SDF-1 this could explain why PA1011 dimerizes.

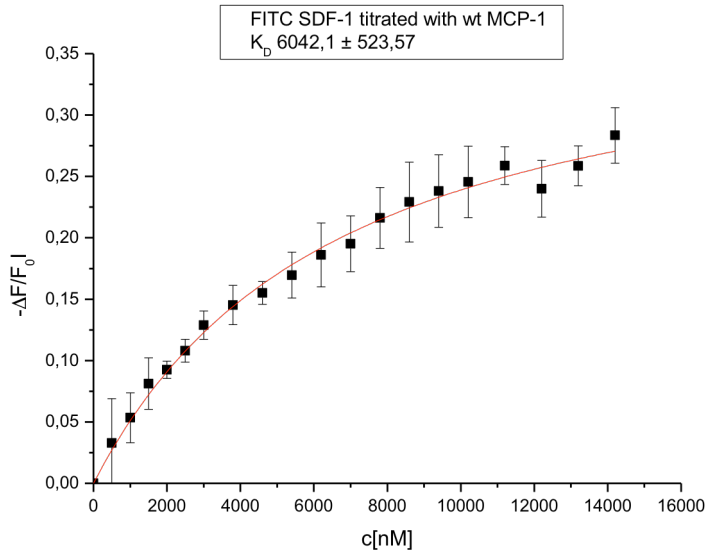


Figure 3.3-7 IFT FITC SDF-1 with wt MCP-1. Labeling with FITC leads to heterophilic interactions of SDF-1. Non-labeled SDF-1 does not interact with any chemokine.

FITC labeled PA1011 furthermore interacts with HSA PA910, wt IL-8, PA910, wt MCP-1 and slightly with

PA401.

The non-mutant counterpart SDF-1 did not interact with any of the chemokines used in this study. FITC labeled SDF-1 however interacted with wt IL-8, PA910, wt MCP-1, as shown in Figure 3.3-7, and slightly with PA1011. In case of the titration FITC SDF-1 with wt MCP-1, the reaction was not saturated applying the usual 500 nM titration steps. Indeed, concentration was increased to 800 nM and 1 µM steps and still saturation was not achieved.

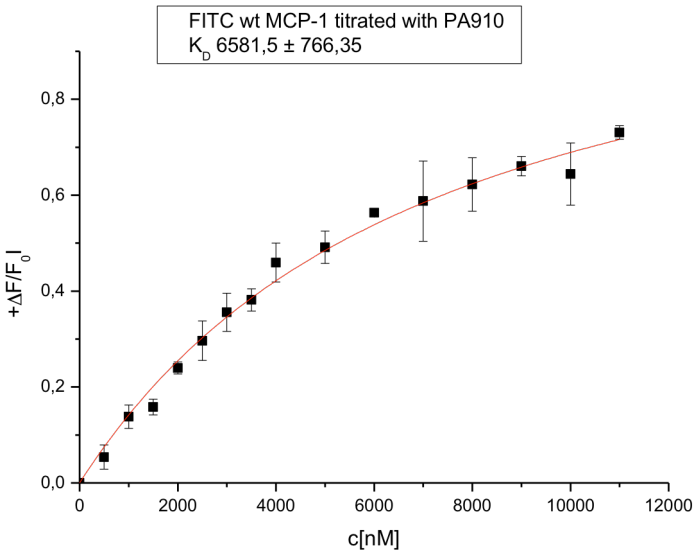


Figure 3.3-8 IFT FITC wt MCP-1 with PA910. Heterooligomer formation occurs only one way.

Titrations were saturation was achieved at only high concentration of the respective ligand occurred especially for reactions involving FITC labeled PA1011, SDF-1 and wt MCP-1 as is evidenced in Figure 3.3-8.

In summary it was found that all chemokines interact heterophilic or homophilic. It was shown that interaction depends highly on whether a chemokine is used as ligand or as labeled protein. It further looks like mutant chemokines seem to have slightly impaired interaction characteristics compared to the respective wild type.
It has been shown that the interaction characteristics of decoy and wild type are not the same. Especially obvious is this result relating to PA910 and wt MCP-1 or met MCP-1; both wild type models interact apparently whereas PA910 does not oligomerize. It has been found in literature that the exchange of one amino acid, Pro[8] to alanine has deep impact on dimerization of MCP-1. In this case the mutant is preserving wild type characteristics but is not able to dimerize, not by itself in solution nor upon interaction with GAGs, even at high concentrations.[68] Therefore a possible explanation why MCP-1 but not PA910 form oligomers could be the deletion or replacement of a dimerization enabling amino acid.
This difference in homooligomerization is true also for PA1011 and SDF-1, although the other way round. As no further investigation was performed in this direction, it can be only assumed

that by generating the decoys, some features influencing chemokine homo- and heterooligomerization could be altered too.
It is also assumed that labeling chemokines with FITC influences the interaction pattern as in many cases e.g. FITC wt IL-8 and FITC PA910 titrated with SDF-1 because interaction appears only one sided.
FITC labels free amine and sulfhydryl-groups so there might be the possibility that in some cases the label interferes with the binding side or leads to a conformational change which again could interfere with the possible interaction of two chemokines.

3.3.2 IFT with FITC labeled chemokines after incubation with heparin

To figure out whether interaction between chemokines is altered or enhanced upon binding to glycosaminoglycans, the ligand chemokine was incubated with heparin. An overview of the results is given in Table 3.3-2 depicting interaction determined in the experiment and if the addition of heparin did change the previous analyzed interaction characteristics.

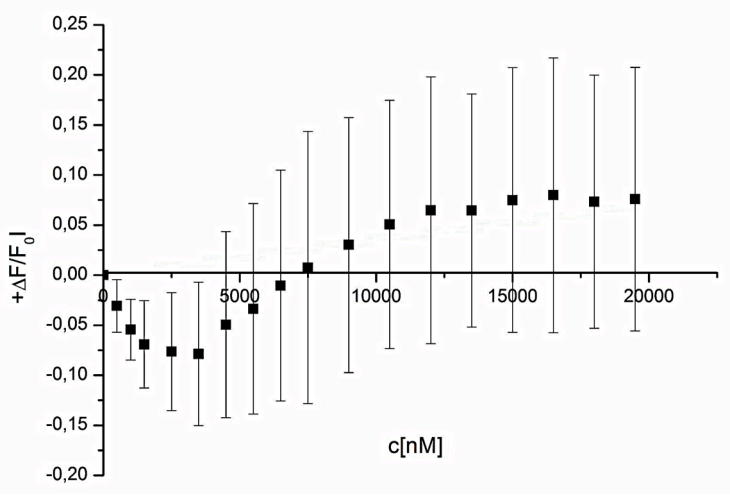


Figure 3.3-9 IFT FITC SDF-1 with PA401 upon incubation with heparin. In this case, it has been shown that previous incubation with heparin can enhance interaction of SDF-1 with PA401. At the beginning, fluorescence is de-quenched and then quenched when the concentration gets about 4 µM.

As some chemokines tend to oligomerize upon binding to heparin like MCP-1 or IP-10 [4] it was expected that the interaction is enhanced. Also SDF-1 is known to dimerize in presence of heparin.[5] Incubation with heparin seems to enhance interaction of FITC PA910 with PA401. The same is true for FITC PA1011 where interaction was strongly enhanced upon previous interaction with heparin and also interaction with FITC SDF-1 is augmented. The biphasic interaction curve of FITC SDF-1 with heparin and PA401 is depicted in Figure 3.3-9. In this case, the fluorescence is de-quenched at the beginning of the titration, presumably a result of specific dimerization reaction and after the concentration is

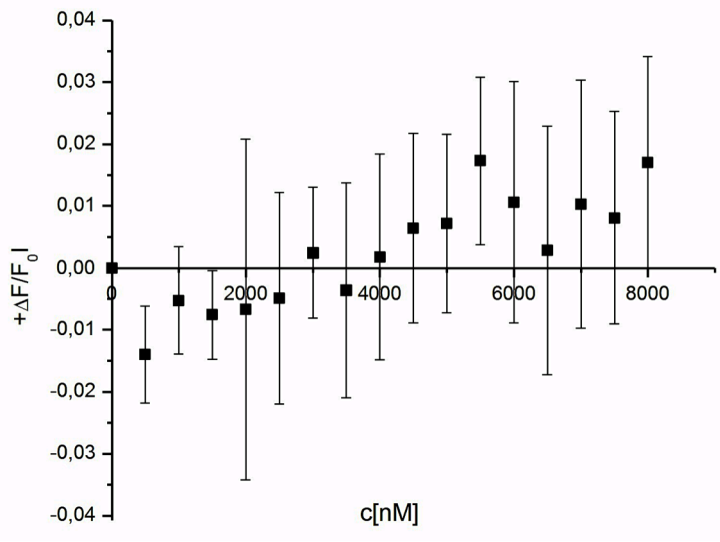


Figure 3.3-10 FITC SDF-1 titrated with PA401. Compared to Figure 3.3--9, prior incubation with heparin enhanced interaction of FITC SDF-1 with PA401. The pattern of fluorescence quenching and de-quenching are the same.

increased to approximately 4 µM the fluorescence is quenched, which may indicate unspecific aggregation. As in the studies without heparin indicated, the fluorescence plotted as $\frac{\Delta F}{F0}$ is also first de-quenched and quenched upon achieving a certain ligand concentration (Figure 3.3-10). Yet in this case, fluoresce quenching occurs when achieving a ligand concentration of ca. 3 µM compared to prior incubation with heparin where quenching occurs at a ligand concentration of 5 µM.

Upon binding to heparin, the interaction of FITC wt IL-8 with PA401 was not enhanced, which might have been possible as binding could enhance the same. In this case either the mutation or the FITC label could disable the interaction or the concentration was too low as under physiological condition IL-8 tends to stay monomeric. As the decoy is generated in a structure and function preserving approach, this might be true also for PA401.
For FITC labeled wt IL-8 and FITC wt MCP-1 addition of heparin did not lead to interaction, which is also the case in the absence of heparin.

Table 3.3-2 An overview of the results obtained by IFT upon incubation with heparin is given.

Protein	Ligand	Interaction	$\pm\frac{\Delta F}{FoI}$.	Prev. interaction
FITC PA910 + Hep	PA401	?	+	-
FITC PA1011 + Hep	**PA401**	**+**	**+**	**?**
FITC SDF-1 + Hep	**PA401**	**+**	**+**	**-**
FITC IL-8 + Hep	PA401	-	-	-
FITC wt MCP-1 + Hep	PA401	-	-	-
FITC PA1011 + Hep	wt IL-8	-	-	+
FITC SDF-1 + Hep	**wt IL-8**	**+**	**-**	**+**
FITC PA401 + Hep	PA910	-	-	-
FITC PA910 + Hep	PA910	-	-	-
FITC PA1011 + Hep	**PA910**	**+**	**-**	**+**
FITC SDF-1 + Hep	**PA910**	**+**	**-**	**+**
FITC PA401 + Hep	**PA1011**	**+**	**-**	**-**
FITC IL-8 + Hep	PA1011	?	-	-
FITC PA910 + Hep	PA1011	-	-	-
FITC wt MCP-1 + Hep	**PA1011**	**+**	**-**	**-**
FITC IL-8 + Hep	SDF-1	-	+	-
FITC PA401 + Hep	SDF-1	?	+	-
FITC PA910 + Hep	SDF-1	-	+	-
FITC SDF-1 + Hep	**SDF-1**	**+**	**-**	**-**
FITC wt MCP-1 + Hep	SDF-1	-	+	-
FITC IL-8 + Hep	**wt MCP-1**	**+**	**-**	**+**
FITC PA1011 + Hep	**wt MCP-1**	**+**	**-**	**+**

Wt IL-8 applied as ligand has shown to not or to not significantly enhance interaction as delineated on the example of FITC PA1011 and FITC SDF-1. FITC PA1011 interacted without previous reaction with heparin with wt IL-8, shown in Figure 3.3-2, after incubation it did not react any longer with wt IL-8, as depicted in Figure 3.3-11.
This phenomenon occurs only in case of FITC PA1011 titrated with wt IL-8. Binding to GAGs could block the binding site necessary to interact with wt IL-8 but as this did not occur while titrating FITC PA1011 and heparin with PA910, wt IL-8 might use a different binding site.
Interaction of FITC SDF-1 with IL-8 is not remarkably enhanced upon incubation with heparin but also it does not have the same impact as for PA1011.
In case of PA910 prior incubation with heparin did not change the results obtained without heparin in any direction, neither enhanced nor decreased interaction. This result would fit the

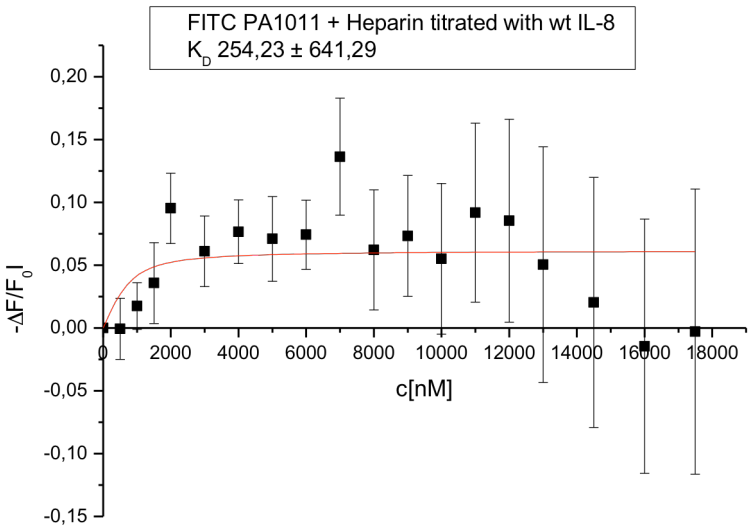


Figure 3.3-11 IFT FITC PA1011 and wt IL-8 with previous incubation with heparin. Without previous incubation with heparin, PA1011 interacted with heparin. Upon incubation with heparin there was no interaction any longer.

theory of the exchange of an amino acid that is crucial for homooligomerization as the wild type, MCP-1 dimerize upon binding to heparin.

Titrating FITC labeled PA401, wt IL-8, PA901 and wt MCP-1 upon heparin incubation with PA1011 leads to interaction with FITC wt MCP-1 and FITC PA401 that do not interact without prior incubation with heparin. The heterophilic interaction of the latter chemokine is depicted in Figure 3.3-12. Further the interaction between FITC wt IL-8 and PA1011 is enhanced. No impact is detected with FITC PA910 interacting still not with PA1011 also the other way round there is interaction with and without heparin.

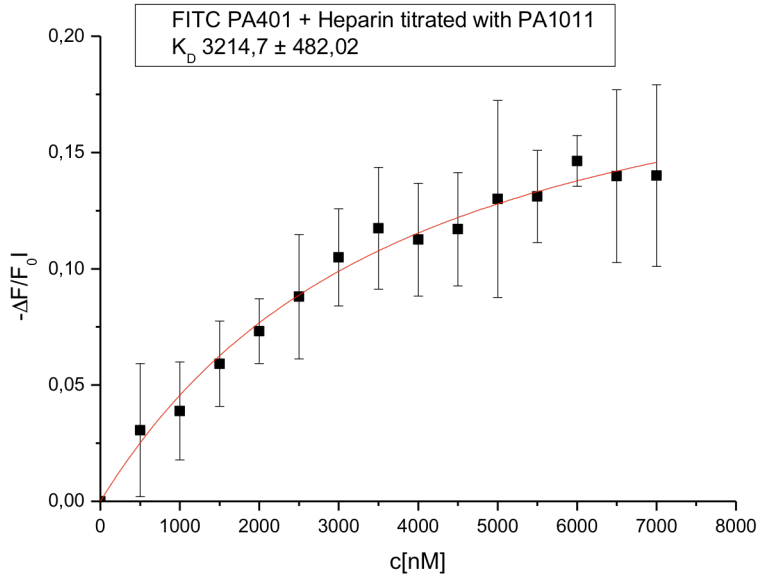


Figure 3.3-12 IFT FITC PA401 and heparin with PA1011. Here, interaction is detectable only upon incubation of PA401 with heparin.

PA401 interacting upon heparin incubation shows that binding to GAGs can lead to chemokine-oligomerization. Without GAG binding PA401 does not or only slightly form heterooligomers, upon binding it interacts also with SDF-1.

Incubating FITC IL-8, PA910 and wt MCP-1 with heparin before titration with SDF-1did not change the result of no previous interaction. Only in case of FITC SDF-1, binding to heparin leads to interaction. This confirms already published results that the monomer-dimer equilibrium shifts towards dimer upon adding a stabilizing counterion such as heparin.[67] Opposite to all

other tested chemokine interaction, the curve was inverted; the maximum was achieved at the beginning and then decreased. A possible explanation might be that the equilibrium shifted back during titration from dimer to monomer.

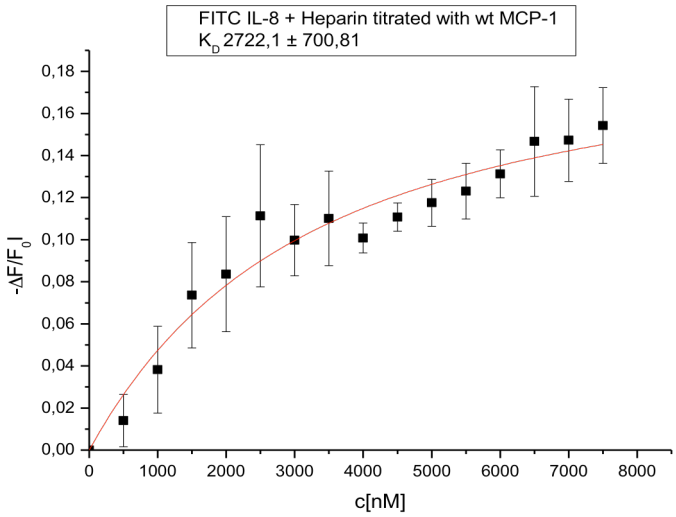


Figure 3.3-13 IFT FITC wt IL-8 and heparin with wt MCP-1. Both chemokines interact also without heparin but interaction upon binding to heparin is enhanced.

Upon incubation with heparin, the interaction of FITC PA401 with SDF-1 was enhanced therefore binding to heparin seems to induce a slight level of oligomerization.

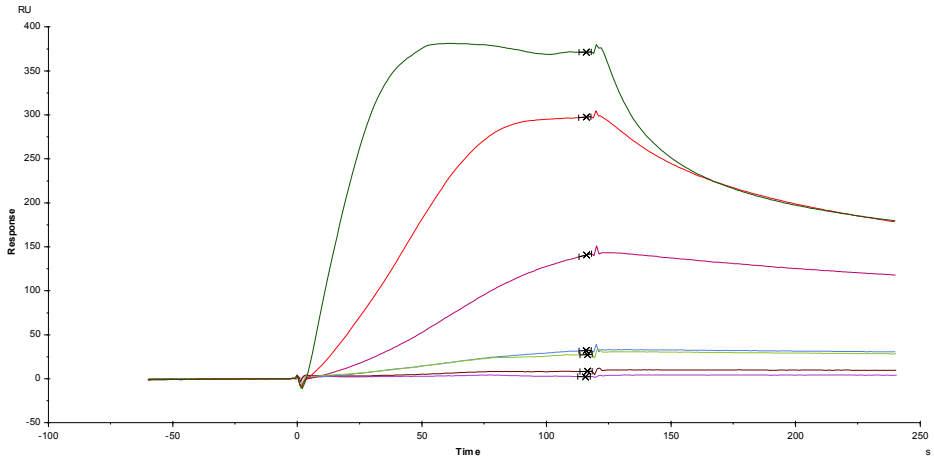
Titrating FITC wt IL-8 and PA1011 upon incubation with heparin with wt MCP-1 leads to an increased interaction and referred to K_D value an increased binding affinity. For PA1011 the K_D decreases from 4101 nM to 188 nM and for wt IL-8 from 13739 to 2722.

In Figure 3.3-13 IFT of FITC wt IL-8 with heparin titrated with MCP-1 is shown. As in other cases, binding to GAG favors oligomerization of the chemokines.

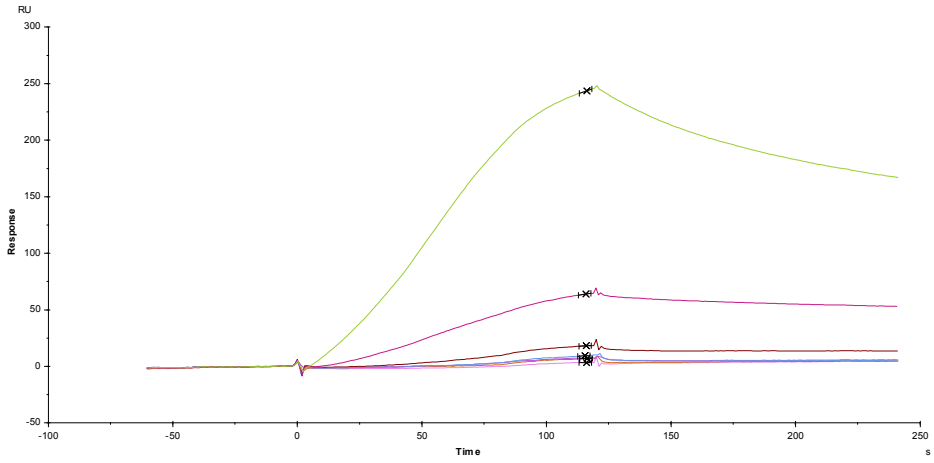
Of the 22 tested potential interactions upon incubation with heparin, in five cases a not previously existing interaction was monitored. In three cases with no previous interaction, at least the interaction seemed to be enhanced, which was the case for the titration of FITC PA910 with PA401, FITC wt IL-8 with PA1011 and FITC PA401 with SDF-1.
In all cases, previous binding to heparin did not decrease previous interaction.

3.4 Surface Plasmon Resonance

To asses interaction of FITC labeled and non-labeled chemokines, SPR was applied. Figure 3.4-1 shows exemplary a sensogram obtained using SPR.



PA1011



FITC PA1011

Figure 3.4-1 An example of a sensogram depicted on the basis of PA1011 and FITC PA1011. As shown in the figure, FITC labeled PA1011 interacts less with heparin compared to the non-labeled chemokine.

The investigated RU and K_D values are illustrated in Table 3.4-1. The evaluation of the data is depicted in Figure 3.4-2 showing clearly the impaired GAG binding affinity of labeled chemokines, in most cases the K_D is the double.

Table 3.4-1 The table illustrates the RU and K_D values determined using SPR. In case of FITC PA401, FITC PA1011 and FITC Met MCP-1, no K_D could be determined.

Chemokine	**R_{max} (RU)**	**K_D [nM]**
FITC PA401		
PA401	896,1	605
FITC wt IL-8	415,5	$221 * 10^3$
wt IL-8	1784,1	$105 * 10^3$
FITC PA1011		
PA1011	758,4	562
FITC SDF-1	1296,6	$160 * 10^3$
SDF-1	819,4	912
FITC PA910	823,3	1808
PA910	484,5	162
FITC met MCP-1		
Met MCP-1	674,3	4989

In Figure 3.4-2 the evaluation of FITC labeled and non-labeled chemokines is presented. There is a significant difference in binding to HS. For all labeled chemokines the binding affinity is way lower compared to non-labeled ones. The exception is PA910 where there is also a difference between labeled and not-labeled chemokine but it is noticeably smaller. For FITC PA401, FITC PA1011 and FITC met MCP-1 it was not possible to determine the K_D, as these data could not be fitted. Therefore the values shown in Figure 3.4-2 were assumed to be high.

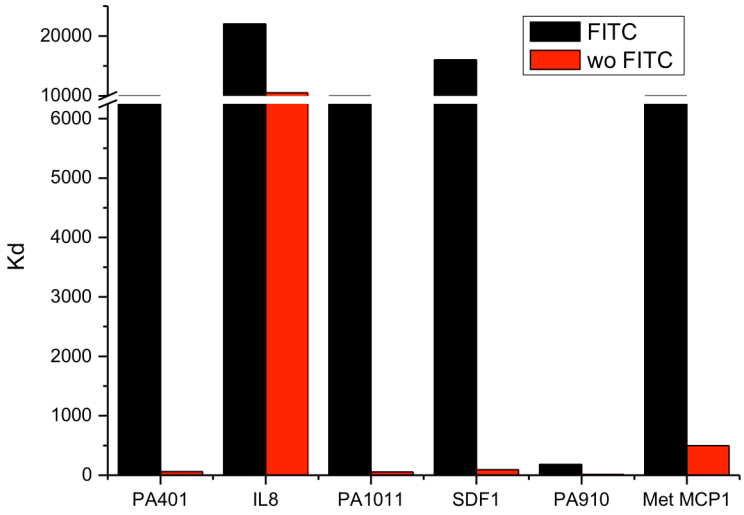


Figure 3.4-2 Data evaluation. The bar chart shows the distinct binding affinity of FITC labeled and non-labeled chemokines. [Bar chart provided by Mag. Tanja Gerlza]

A possible explanation could be that the protein concentration was too low and only the linear response phase was measured as no saturation was achieved. In this case the field-tested concentrations, suitable for non-labeled proteins have to be determined for those with a FITC label. As also assumed in IFT experiments, interaction seems to be influenced by the FITC label, which could explain the need for a different FITC protein concentration necessary to determine K_D value in SPR. According to literature, the protein concentration range for analyte injection should span 10-fold above and below the expected K_D. and the analyte concentration must be known precisely.[69]

In this case FITC influenced the interaction of chemokines with GAGs but it can also be true for chemokines interaction among each other. Moreover compared to IFT, SPR is a more reliable approach to investigate impact of FITC labeling. IFT has to be performed in a very accurate and steady manner that might lead to mistakes if not performed properly.

4 Conclusion

Little is known about chemokines interacting with each other. From the applied chemokines used in these experiments, IL-8, MCP-1 and SDF-1 are known to form heterodimers with PF-4, MCP-1 with MCP-4, MCP-2 and eotaxin and SDF-1 with NAP-1[25,45]
In our study we have tried to figure out which chemokines do interact with other chemokines in a homo- or heterophilic way. As gel shift assays and western blots turned out to be unsuitable approaches to detect this interaction, the results are based on observations obtained by IFT. Applying this method, it was not possible to give clear answers about all interactions; only whether or not two distinct chemokines are interacting can be investigated, together with a good estimation of the K_D value. This method is therefore the first step into the right direction to continue research of chemokine interactions.
Many chemokines tend to dimerize upon achieving a certain concentration or receptor binding but as has been shown all of the applied chemokines also do interact heterophilic with other chemokines already in solution. Heterooligomerization is found to be initiated and enhanced in a few cases upon binding to heparin.
Interestingly the tendency to dimerize in solution is found to be not distinctive, which is surprising, as IL-8 and MCP-1 are known to form dimers. SDF-1 otherwise is monomeric even at high concentrations but is able to form dimers under certain conditions. As proven, these conditions were achieved during our experiment.
Noticeably are the different interaction characteristics of decoy and wild type chemokines. These were expected to be similar as only non-crucial amino acids were replaced to generate the mutants.
Also an important factor seems to be the FITC label. The experiment shows that there is a significant divergence if a chemokine is labeled and used as ligand or not-labeled and used as titrant. Therefore it could be useful to try other labeling methods, as a common problem with labeling chemokines is the possible affection of reaction kinetics and sensitivity caused by a fluorescent label.

The impact of labeling is further indicated in the SPR experiment. As confirmed by investigating the different binding affinity of labeled and non-labeled chemokines it has been clearly shown that the label diminishes the binding affinity towards HS immense. This could have been influenced by the inaccuracy of protein concentration determinations due to FITC label.

Clearly the determination of oligomerization is necessary to provide a further understanding in chemokine-mediated processes. Targeting chemokine interactions rather than only chemokines might provide an appealing approach for therapeutic intervention.

5 Future Aspects

This research indicates that all of the tested chemokines can form heterooligomers, which might offer a working point for therapeutically approaches. As interaction of chemokines was tested with only one method, further research in this direction is necessary. First of all changes in interaction pattern and protein concentration based on FITC labeling have to be excluded. To obtain reliable results, all positive interaction should occur in both directions to avoid label-induced aberrances. For further testing the occurring interactions fluorescence anisotropy could be a feasible method. If the interaction is confirmed more accurate methods as NMR or mass spectrometry can be applied.

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