THE AFFINITY PRECIPITATION FOR THE ISOLATION OF BIOMOLECULES

THÈSE N^{O} 3862 (2007)

PRÉSENTÉE LE 31 AOÛT 2007

À LA FACULTÉ DES SCIENCES DE BASE

Institut des sciences et ingénierie chimiques

SECTION DE CHIMIE ET GÉNIE CHIMIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

PAR

Gisela STOCKER MAJD

ingénieure chimiste diplômée EPF

de nationalité suisse et originaire de Wädenswil (ZH)

acceptée sur proposition du jury:

Prof. P. Vogel, président du jury

Prof. R. Freitag , directrice de thèse

Dr M. Costioli, rapporteur

Prof. H. Lashuel, rapporteur

Dr I. Petrescu, rapporteur

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

Lausanne, EPFL

2007

A mi querida Madre

Remerciements

Cette thèse a aboutie grâce au soutien indéniable de plusieurs personnes. Je tiens ici à leur présenter ma plus grande gratitude. Un grand merci :

A ma directrice de thèse, Prof. Ruth Freitag, pour m'avoir acceptée dans son laboratoire et pour m'avoir judicieusement orientée et conseillée pendant les moments difficiles de ma thèse.

A Caroline Vandevyver, pour son aide inestimable, ses conseils précieux et sa disponibilité.

A Frank Hilbrig et Igor Fisch pour leur savoir-faire.

A Claudia Bossen et à Karim Schubiger pour leur aide, et tout particulièrement à Dorothée Dumoulin, pour sa contribution primordiale dans le projet de phage display.

A mes collègues du LBCH pour leur amitié, leur collaboration, et la magnifique ambiance: avec le « groupe des filles » Marilia, Magda et Vera, et le « groupe des garçons » Arnaud, Matteo, Stéphane et Kevin.

A toutes les apprenties qui sont venues travailler dans le laboratoire, Noémi, Valérie et Vanessa.

A tous les collaborateurs, anciens et nouveaux, des groupes Wurm et Mermod, tout particulièrement à Jean, Lucia, Luisa, Ione, Mougli et David.

Aux collègues des autres laboratoires, Guillaume Alain Razaname, Robert Driscoll et Otto Hagenbüchle, qui m'ont aidée avec différentes analyses.

Aux membres du jury de thèse, pour avoir accepté d'apporter leurs critiques et d'examiner ce travail.

Au Fonds National Suisse pour le financement du projet.

A Catherine Vinckenbosch, du Service Social de l'EPFL, pour son soutient.

A Fatima Gueroui pour le temps passé à corriger le texte dans la langue de Molière.

A tous mes amis pour leur présence et encouragements.

A ma famille en Suisse, au Panama et au Maroc, qui m'ont énormément soutenue pendant ce travail.

A Hicham, mon cher mari, pour son aide précieuse, son soutien et son amour.

Finalement, à Hakim, mon magnifique petit garçon, qui est ma joie et ma motivation.

Abstract

Product isolation increasingly becomes a major bottleneck in molecular biotechnology. This is especially the case for the sensitive proteineous substances (recombinant proteins, antibodies) and lately also DNA, usually produced in low concentration. The current challenge to preparative bioseparation can be summed up as the need to perform an economically sound, high-resolution separation at large scale, while maintaining "physiological" conditions throughout. Currently the only approach, which allows the direct, selective enrichment of the product from a highly diluted and complex feed, is to use biospecific interactions, i.e. "affinity" techniques. Affinity chromatography, the best known and understood of these methods may not be ideally suited early on during the downstream process because of danger of column fouling with most raw feeds, but also because of limited scale up potential and high costs (equipment, material). A promising technique combining the affinity interaction and the precipitation technique, called affinity precipitation, significantly increases the selectivity while retaining the main advantages of the precipitation method like the relatively simple equipment requirements and the applicability at large industrial scale. Affinity precipitation should also have advantages in terms of scalability, handling, and costs.

In affinity precipitation, the ability of certain water-soluble polymers to form a separate phase under particular environmental conditions is used to isolate and purify biomolecules. The precipitation requires only small changes in the environment (pH, ionic strength, temperature, light, presence of specific substances). Such polymers are often called "stimuli-responsive" polymers. PolyNIPAAm is one of the most studied thermoresponsive polymers. Such molecules typically show a critical solution temperature (CST) in aqueous solution, i.e. they are soluble in cold water, but become insoluble and precipitate once the CST is surpassed. The phenomenon is usually fully reversible and the molecules redissolve readily once the temperature is lowered again. Thermoresponsive bioconjugates carrying an affinity ligand have been suggested for the specific purification of biologicals by affinity precipitation. The involved bioconjugate is called an affinity macroligand, AML. The polymer mediates the response to the stimulus (e.g. a change in temperature). The affinity tag enables the AML to attach itself to the target molecules.

Affinity precipitation is to date not an established downstream processing technique in the biotech industry. The objective of this thesis was to find potential applications for the affinity precipitation by first choosing interesting target molecules and second by comparing the affinity precipitations with well established affinity bioseparation procedures based on chromatography or magnetic beads. The biomolecules chosen as target products were poly(A) mRNA, scFv antibody phages, hemoglobin and IgG 4E11. To my knowledge, it is the first time that these biomolecules have been isolated using the affinity precipitation technique.

The first application, chapter 2, is about the purification of RT-PCR competent poly(A) mRNA from crude cell lysate by affinity precipitation. An AML-precursor consisting of avidin covalently linked to polyNIPAAm was used for the recovery of poly(A) mRNA hybridised to biotinylated poly(dT)-tags from crude cell lysates (Jurkat cells) by affinity precipitation. The results of the affinity precipitation were compared to those achieved with an accepted standard purification of poly(A) mRNA using avidin-activated magnetic beads. Both yield and quality / purity of the affinity precipitated poly(A) mRNA were found to be similar or better (especially removal of rRNA) than for poly(A) mRNA prepared by the magnetic particle-based protocol, while both mRNA-isolates performed equally well in standard reverse transcriptase amplification (RT-PCR) of a β actin transcript fragment.

The second application, chapter 3, is about the screening of a synthetic human antibody phage display library against the MUC-1 surface antigen using smart bioconjugates. A synthetic antibody phage library (ETH-2) was screened against the MUC-1 peptide. Three methods were used, immuno tubes coated with the peptide antigen, streptavidin coated paramagnetic particles together with a biotinylated peptide, and a stimuli-responsive conjugate of avidin and polyNIPAAm. In combination with the biotinylated MUC-1, the avidin-polyNIPAAm conjugate was used to isolate specific phages from the library in several rounds of panning, consisting of repeated thermoprecipitation / redissolution cycles. Compared to the established techniques, affinity precipitation of the phages led to a greater variety of genetically different specific phage antibodies.

The third application, chapter 4, is about the use of human haptoglobin-polyNIPAAm as AML for affinity precipitation of human hemoglobin. Affinity precipitation was included as the final purification step in a hemoglobin isolation protocol from blood. The first steps of the process were realized by traditional methods (lysis of red blood cells and protein precipitation with ammonium sulphate). Five different haptoglobin-polyNIPAAm AML were constructed changing the polymer:haptoglobin coupling ratio. Affinity precipitation was compared to affinity chromatography and batch adsorption methods using two binding / elution protocols. The harsh elution conditions (one at pH below 2, the other with 5 M urea) needed for dissociation of the hemoglobin-haptoglobin complex rendered the recycling of the haptoglobin AML difficult. Nevertheless, the affinity precipitation was a suitable method for the purification of hemoglobin. The results obtained with these AML confirm the values obtained by others using current methods in regard to binding ratio between haptoglobin and hemoglobin and the association constant (in the expected range).

The four application, chapter 5, is about the use of affinity precipitation for antibody purification. To reach this goal, five AML were constructed and tested. The best results were obtained with the Protein A-AML, with nearly the expected ratio as found in the literature. Two approaches were used for the construction of the AML and it was observed that the direct coupling of polyNIPAAm reduced the binding ratio (blocking of the binding sites responsible for the affinity interaction) compared to the AML obtained by an avidin-biotin interaction, which apparently bypasses this problem. However this last AML is bigger and more costly. A Protein A mimic was also tested in affinity precipitation but the results weren't satisfactory although not worse than those for affinity chromatography using this ligand. FLAG-tag peptide was tested in affinity precipitation / chromatography for IgG 4E11 purification but again no satisfactory antibodies recovery was obtained. It seems that stressed (partly denatured) antibodies were used, affecting the interaction between capture molecules and IgG, but also giving an underestimation of the ELISA results. However, the first results with affinity precipitation of antibodies are promising but need to be further optimization in regard to the elution conditions and the quantification methods.

Key words: bioseparation, affinity precipitation, critical solution temperature (CST), thermoresponsive materials, poly-N-isopropylacrylamide (polyNIPAAm), affinity macroligand (AML), thermocycling step, RNA purification, MUC-1 (mucin), phage display, haptoglobin, hemoglobin, antibody, Protein A, FLAG-tag, avidin, biotin.

Résumé

La purification de biomolécules devient une des étapes clés dans les processus biotechnologiques, car ces composés sont souvent produits à faible concentration et dans des milieux très complexes à purifier. Par ailleurs, une pureté et une qualité très élevées sont nécessaires pour les produits destinés aux domaines tels que pharmaceutiques ou médicaux. D'où l'intérêt de développer des méthodes très performantes de purification, mais qui n'affectent pas les propriétés des produits purifiés. Des méthodes exploitant l'interaction par affinité entre une molécule de capture et sa molécule cible, sont très prometteuse pour atteindre ce but. La chromatographie par affinité est une des méthodes les plus connues, mais elle ne peut pas s'appliquer quand le mélange à purifier contient des composants qui pourraient bloquer la colonne. Cette methode présente aussi des limites pour faire un scale-up du processus. D'autre part, la précipitation par affinité est une méthode très prometteuse car elle réuni la capacité de reconnaître et de capturer une molécule cible par affinité, puis de concentrer celle-ci après précipitation et séparation des autres molécules du mélange. De plus, l'équipement qui lui est nécessaire est simple et s'adapte aux processus industriels.

Le polyNIPAAm, un des polymères thermosensibles les plus connus, se caractérise par son comportement en solution aqueuse face aux changements de température. En dessous d'une température critique de solubilité (CST), le polymère est soluble, mais en dessus il se replie et forme des agrégats qui précipitent. Un Macroligand d'Affinité (AML) peut être obtenu en attachant ce type de polymère à une molécule de capture. Par la suite, l'AML sera utilisé pour la purification de la molécule cible présente dans un mélange, en utilisant la précipitation par affinité.

Actuellement, la précipitation par affinité n'est pas utilisée dans l'industrie. L'objectif de cette thèse est double. Premièrement, il s'agit de trouver des applications potentielles avec des biomolécules intéressantes. Deuxièmement, réaliser des comparaisons avec des méthodes déjà bien établies comme la chromatographie par affinité, l'adsorption par affinité en batch (billes magnétiques ou billes de sepharose) ou l'adsorption par affinité sur immunotubes. Les molécules cibles choisies sont le poly(A)mRNA, les anticorps scFv exprimés sur des phages et l'hémoglobine. A ma connaissance, c'est la première fois que ces molécules ont été purifiées en ayant recours à la précipitation par affinité.

Le poly(A) mRNA a pu être purifié en partant du surnageant obtenu après la lyse des cellules Jurkat. L'AML précurseur était constitué de l'avidine bioconjuguée au polyNIPAAm. La capture de la molécule cible s'est fait d'abord par la formation du complexe biotine-oligo d(T) / poly(A) mRNA, puis l'AML précurseur a été ajouté en formant la très forte liaison non-covalente entre l'avidine et la biotine. La méthode a été comparée avec l'adsorption par affinité en batch en utilisant la streptavidine couplée aux billes magnétiques. Des tests de pureté et de qualité ont confirmé que la méthode est adéquate et qu'elle permet un meilleur rendement de poly(A) mRNA purifié comparé aux billes magnétiques. Après purification, le mRNA a pu être utilisé pour d'autres analyses tel que des RT-PCR.

Une librairie de phages présentant des anticorps scFv a été balayée afin d'obtenir des anticorps capables de reconnaître le peptide MUC-1. Ce peptide est présent à l'extérieur de certaines cellules cancéreuses et pourrait être utilisé comme cible pour des traitements anti-cancer. Un peptide MUC-1 biotinylé a été fixé par liaison non-covalente à l'AML précurseur (avidine bioconjuguée au polyNIPAAm). L'AML a été utilisé pour capturer les anticorps scFv sur phages capables de reconnaître le MUC-1, en faisant deux cycles de sélection.

L'ADN des phages sélectionnés a été analysé avec différentes méthodes puis séquencé pour trouver des similitudes. C'est la première fois qu'un composé aussi grand (particule virale) a été capturé par un AML. La précipitation par affinité a été comparée avec l'adsorption par affinité en batch (billes magnétiques) et avec l'adsorption par affinité sur immunotubes. Une plus grande diversité des anticorps a été trouvée en utilisant la précipitation par affinité avec deux possibles epitopes sur le MUC-1, ce qui n'a pas pu être déterminé avec les deux autres méthodes.

La précipitation par affinité a été introduite comme l'étape finale dans la purification de l'hémoglobine suivant un protocole pour l'extraire du sang. Les premières étapes du protocole utilisaient des procédés traditionnels pour casser les globules rouges du sang puis faire une précipitation fractionnée du mélange avec le sulphate d'ammonium. Cinq différents AML ont été construits en faisant varier le rapport du couplage entre le polymère et l'haptoglobine. La précipitation par affinité a été comparée avec, d'une part, la chromatographie par affinité et, d'autre part, avec l'adsorption par affinité en batch en utilisant l'haptoglobine couplée à des billes de sepharose. Les deux protocoles utilisés ont eu recours à des conditions d'élution très agressives (l'un avec pH 2, l'autre avec 5 M urée) pour dissocier le complexe hémoglobine-haptoglobine, et il n'a pas été possible de recycler l'AML dans ces conditions. Malgré cela, la précipitation par affinité reste une méthode utilisable pour la purification de l'hémoglobine car les résultants obtenus par ces AML (le nombre molécules d'hémoglobine capturée par haptoglobin (AML) et la constante d'affinité entre eux) sont en accord avec les résultats obtenus dans la littérature.

L'obtention d'anticorps thérapeutiques purs et en quantité suffisante représente un grand défi pour les méthodes de purification. La précipitation par affinité a été utilisée pour purifier des IgG grâce à cinq AML construits. Les meilleures résultats ont été obtenus en utilisant la Protéine A (Protein A-AML) comme molécule de capture. Deux approches ont été utilisées pour la construction des AML contenant la Protéine A. Il a été observé que lorsque le polyNIPAAm est directement couplé avec la Protéine A, l'efficacité de l'AML est réduite. Ce résultat provient probablement du blocage des sites sur la Protéine A, ces derniers étant responsables de l'interaction avec l'anticorps. Ce problème semble être évité quand l'AML est construit en utilisant l'interaction avidine-biotine. Par contre, ce dernier AML est plus volumineux et plus cher. Un autre AML contenant un mimétique de la Protéine A a aussi été construit et testé avec la précipitation par affinité, mais les résultats n'ont pas été satisfaisants. Néanmois, ces résultats sont similaires à ceux obtenus avec la chromatographie d'affinité. Le peptide FLAG-tag a aussi été utilisé dans la construction des AML puis testés par précipitation et chromatographie d'affinité pour purifier des IgG 4E11. Malheuresement, les résultats demeurent également non satisfaisants avec ces méthodes. Il est probable que des anticorps dénaturés aient été utilisés, ce qui a affecté l'interaction entre les molécules de capture et l'IgG. Ce qui a encore produit une sous-estimation des résultats obtenus par le test ELISA. Toutefois, ces premiers résultats obtenus, en utilisant ces différents AML avec la précipitation par affinité, sont prometteurs, mais il demeure nécessaire d'optimiser les conditions de capture et d'élution ainsi que la méthode de quantification.

Mots clés: bioséparation, précipitation par affinité, température critique de solubilité (CST), polymère sensible à la température, poly-N-isopropylacrylamide (polyNIPAAm), MacroLigand d'Affinité (AML), étape de précipitation et centrifugation en dessus de la CST, ARN messager, MUC-1 (mucine), présentation sur phage, haptoglobine, hémoglobine, anticorps, Protéine A, étiquette FLAG, avidine, biotine.

Contents

1. General introduction

1.1 Affinity techniques for bioseparation

Product isolation increasingly becomes a major bottleneck in molecular biotechnology. This is especially the case for the sensitive proteineous substances (recombinant proteins, antibodies) and lately also DNA, usually produced in low concentration. Since these bioproducts have to be purified from a complex production environment, high-resolution bioseparation techniques are required [1]. The downstream process (DSP) is where the majority of the production costs are created, while between 60 % and 80 % of the (high value) product is lost [2, 3]. The current challenge to preparative bioseparation can be summed up as the need to perform an economically sound, high-resolution separation at large scale, while maintaining "physiological" conditions throughout [4].

The isolation of a high value product is governed by two, somewhat antagonistic aspects, that of "concentration" and that of "isolation". Currently the only approach, which allows the direct, selective enrichment of the product from a highly diluted and complex feed, is to use biospecific interactions, i.e. "affinity" techniques [5]. A number of well-known separation operations have been improved in terms of selectivity by introduction of the "affinity motif". Examples of such techniques are, affinity chromatography, fluidized bed affinity adsorption, affinity extraction in aqueous two-phase systems, membrane affinity filtration, and last but not least, affinity precipitation [6-10].

An important aspect of the early stages of the DSP is the removal of water. This reduces the process volume for the subsequent stages, which renders the overall process considerably more economic [4]. Affinity separations are well suited for such concentration steps. However, affinity chromatography, the best known and understood of the affinity methods may not be ideally appropriate early on during the DSP because of danger of column fouling with most raw feeds, but also because of limited scale up potential and the high costs (equipment, material) associated with this method [8].

Precipitation techniques (using ammonium sulphate, polyoxyethylene, organic solvents) are among the most attractive industrial concentration methods because they require only simple equipment and are easily adapted in scale. Their main disadvantage is the low selectivity and,

as a consequence, the low degree of obtainable purification [10]. A process combining the affinity interaction and the precipitation technique, i.e., affinity precipitation, significantly increases the selectivity of the method and retains the above – mentioned main advantages of the precipitation method like the relatively simple equipment requirements and the potential for scale-up.

1.2 Affinity precipitation

At present, two principle types of affinity precipitation are known [7, 8]. The primary effect affinity precipitation, also called the homobifunctional variant (figure 1.1), uses an at least bivalent ligand to precipitate a multivalent target molecule.

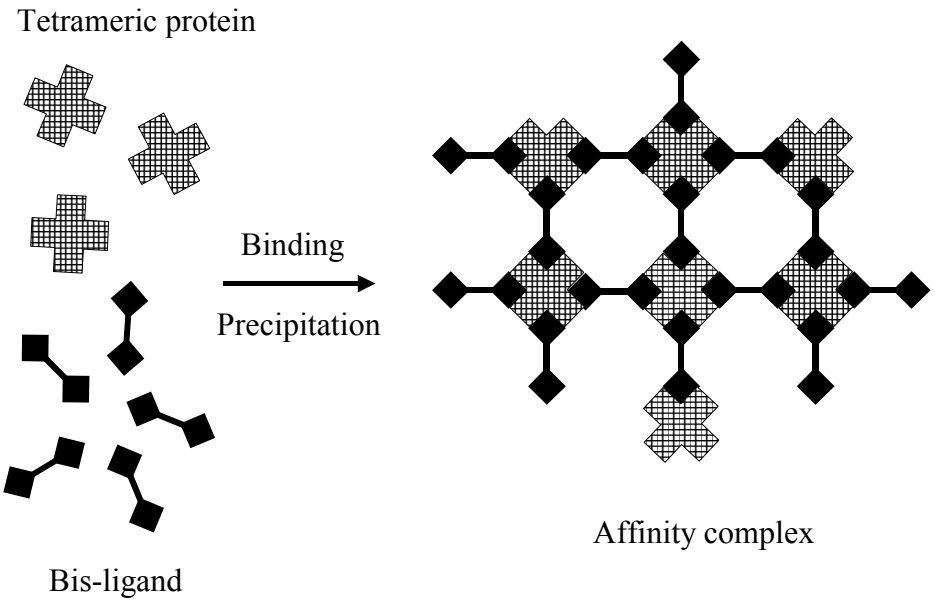


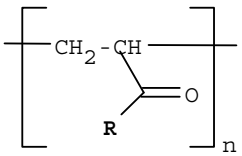
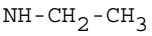
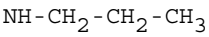
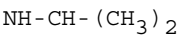
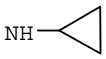
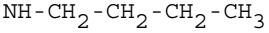
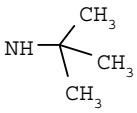
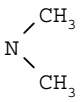
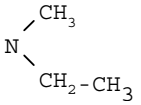
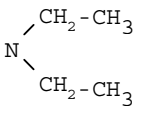
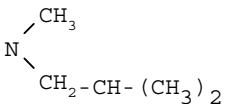
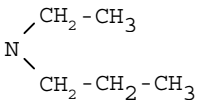
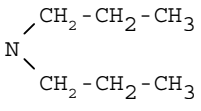
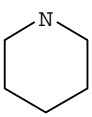
Figure 1.1. Principle of primary effect affinity precipitation using a bis-ligand as cross-linker to precipitate a tetrameric protein

If the ligand:target molecule ratio is close to equivalence, large networks form and the complex precipitates. Primary effect affinity precipitation has disadvantages like needing to know the approximate concentration of the target molecule before adding the ligand, and difficulties in redissolution of the precipitate and recovery of the target molecule. The method is also time consuming and restricted to ligands and products that carry at least two binding sites.

Secondary effect affinity precipitation, also called the heterobifunctional variant (figure 1.2), only needs a single interaction site on the affinity ligand and on the target product, and is characterized by an independency between affinity interaction and precipitation. Here, the ability of certain water-soluble polymers to form a separate phase under certain environmental conditions is used to isolate and purify biomolecules. The precipitation takes place when small changes in the environment occur (pH, ionic strenght, temperature, light, presence of specific substances). Such polymers are often called "smart" or "stimuli-responsive" polymers. Salt- and pH-responsive polymers typically bear charges and hence show a certain tendency for non-specific interaction with biologicals such as proteins and polynucleotides. Thermoresponsive polymers such as poly(N-isopropylacrylamide), polyNIPAAm, on the other hand, show little tendency for interaction with biologicals unless activated with a specific interactive affinity ligand [11].

PolyNIPAAm is one of the most studied thermoresponsive polymers [12]. Such molecules typically show a critical solution temperature (CST) in aqueous solution [13], i.e. they are soluble in cold water, but become insoluble and precipitate once the CST is surpassed (see table 1.1). The phenomenon is usually fully reversible and the molecules redissolve readily once the temperature is lowered again [14, 15]. In the case of polyNIPAAm a critical solution temperature between 32 °C and 34 °C has been reported in pure water [13, 16]. The CST changes as a function of the composition of the aqueous solution and especially salts tend to lower the CST [17, 18]. Precipitation (and redissolution) is sudden and in the case of polyNIPAAm occurs over a small (< 2 °C) temperature interval.

Table 1.1. CST of aqueous solutions of poly (N-substituted acrylamides) from references [12, 19-21]

 **R**	**Poly(N-alkylacrylamide)**	**CST (°C)**
	Poly(acrylamide)	soluble
	Poly(N-methylacrylamide)	soluble
	Poly(N-ethylacrylamide)	82
	Poly(N-n-propylacrylamide)	22
	Poly(N-isopropylacrylamide)	32-34
	Poly(N-cyclopropylacrylamid)	47
	Poly(N-n-butylacrylamide)	insoluble
	Poly(N-t-butylacrylamide)	insoluble
	Poly(N,N-dimethylacrylamide)	soluble
	Poly(N-ethyl, N-methylacrylamide)	56
	Poly(N,N-diethylacrylamide)	32-42*
	Poly(N-isopropyl, N-methylacrylamide)	25
	Poly(N-ethyl, N-propylacrylamide)	insoluble
	Poly(N,N-dipropylacrylamide)	insoluble
	Poly(N-acryloylpiperidine)	55
	Poly(N-acryloylpyrrolidine)	4

* Depending on the tacticity of the polymer

Thermoresponsive bioconjugates carrying an affinity ligand have been suggested for the specific purification of biologicals by affinity precipitation [11]. The involved bioconjugate is called an affinity macroligand, AML. A typical AML consists of a stimuli-responsive polymer to which an affinity ligand is linked. The polymer mediates the response to the stimulus (e.g. a change in temperature). The affinity tag enables the AML to attach itself to the target molecules (capturing step).

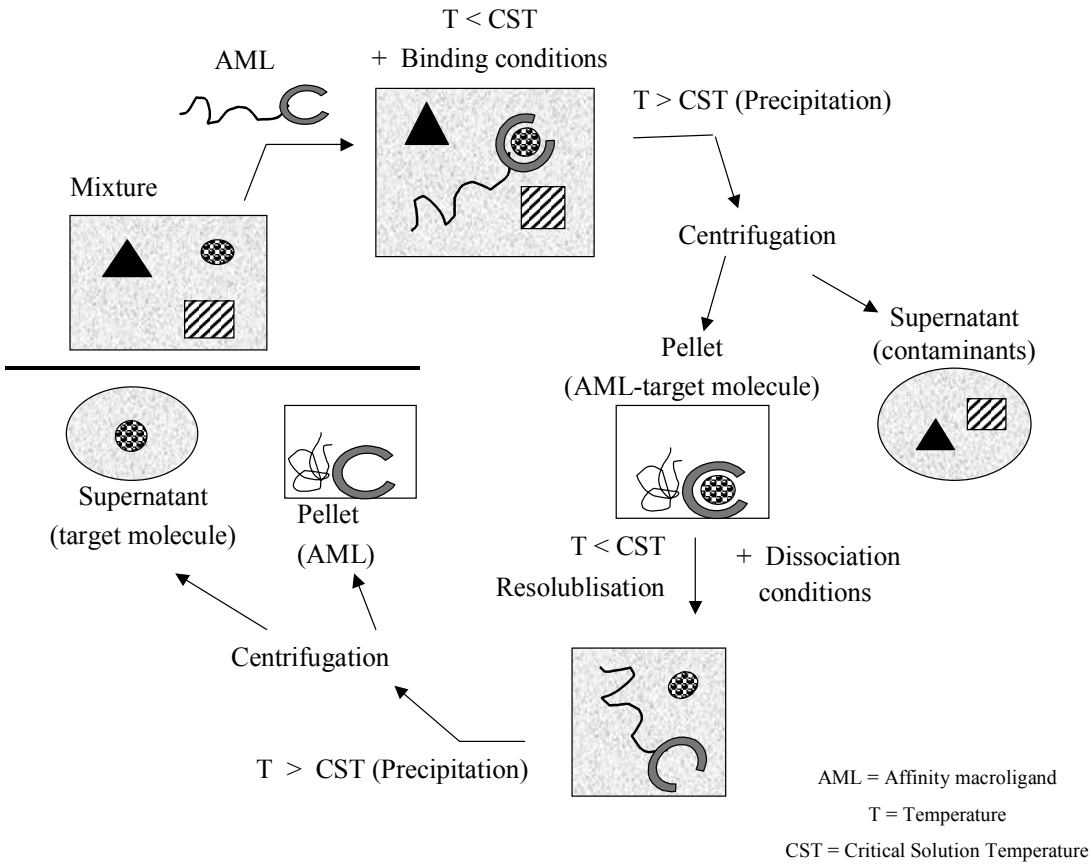


Figure 1.2. Principle of secondary effect affinity precipitation. A heterobifunctional affinity macroligand (stimuli-responsiveness, affinity interaction) is used to precipitate a monovalent target molecule.

In figure 1.2, the general procedure for the affinity precipitation used in this thesis is described. Briefly, affinity interactions between the AML and the target molecules occur in free solution below the CST before inducing the precipitation by an increase of the temperature above the CST. Contaminants remain in solution and they are removed after a thermocycling step (precipitation/centrifugation). If a more thorough elimination of the contaminants is required, washing steps under binding conditions can be done at this point (by repeatedly precipitating and redissolving the affinity complex in fresh binding buffer). To elute the product from the complex, the pellet containing the precipitate is redissolved in

dissociation / elution buffer below the CST. The finally recovery of the target molecule and removal of the AML is achieved by thermoprecipitation of only the AML under non-binding (elution) conditions. The product stays in solution under these circumstances.

The state-of-art in method development and instrumentation in bioseparation via affinity precipitation has recently been summarized [22]. The method is robust and easily adapted to various scales and formats. The precipitate can be recovered by centrifugation, as described above, but also by filtration. Since only liquids are involved, while the solid precipitate can be produced and redissolved in a controlled manner whenever desirable, the method is especially suited for applications where the transport and handling of solids is considered cumbersome compared to that of liquids, which can be pumped and injected.

A number of cases studied can be found in the pertinent literature, where affinity precipitation is used for the isolation or enrichment of biologicals. Some of these cases are compiled in ref. [8]. For example, polyNIPAAm was used for the construction of different AML to target molecules like trypsin [23], α-amylase inhibitor [24], restriction endonuclease (*hind* III) [25], rabbit C-reactive protein [26] or human IgG [27]. Our group has recently investigated molecules like histidin-tagged and APP-tagged fusion proteins [11], plasmid DNA [28], were also tested as target molecules for applications with polyNIPAAm-containing AML. The affinity precipitation method studied in this thesis, was a secondary effect affinity precipitation; the thermoresponsive polymers used to prepare the AML was polyNIPAAm.

1.3 PolyNIPAAm and CST phenomenon

Figure 1.3 shows the structure of polyNIPAAm used for the affinity precipitation in this thesis. The polymer was provided by the LBCH group (EPFL) and was synthesized by telomerization (chain transfert polymerization) of the monomer (N-isopropylacrylamide) with a telogen (chain transfer agent, 3-mercaptopropionic acid), and a radical starter (2,2'-azoisobutyronitrile) in organic solvent as described by Chen and Hoffman [29]. This polymerization method produces a particularly interesting polymer as the structure is homogeneous showing a polydispersity below 1.2 [5]. Other advantages are the small size of this polymer (oligomer, Mw < 5000 g/mol), and the presence of one carboxylated reactive end-group, allowing the introduction of the affinity ligand specifically at the terminus of the molecule [28, 30].

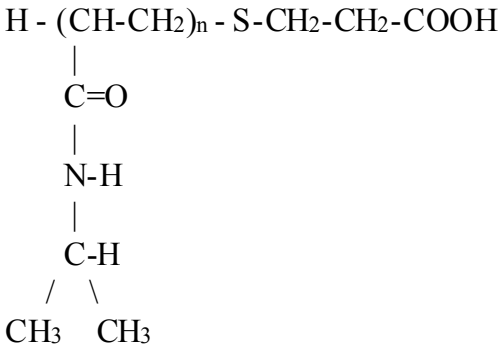


Figure 1.3. Structure of the carboxylated poly(N-isopropylacrylamide) used to prepare the AML .

PolyNIPAAm is an uncharged reversibly water-soluble polymer. Its solubility depends strongly on the temperature and the occurrence of a critical solution temperature (CST), above which precipitation occurs (figure 1.4) is in pure water typically observed between 32 °C and 34 °C for the concentration range of 1-5 % (w/v) [31, 32]. The presence of the affinity ligand in the AML or the formation of the complex with the target product usually has no influence on the CST of the polyNIPAAm [33-35]. The fact that an aqueous solution of polyNIPAAm undergoes a sharp phase transition around the physiological temperature has certainly contributed to making it one of the preferred temperature sensitive polymer to study.

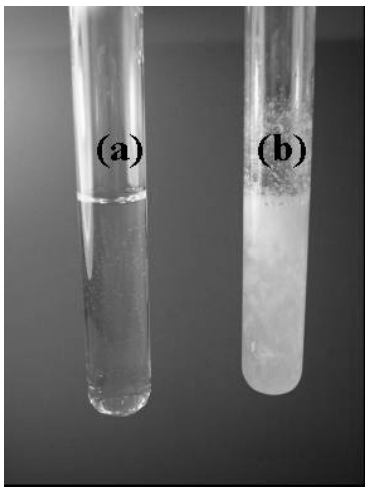


Figure 1.4. Source: [5]. Phase separation of polyNIPAAm in water. (a) Below the CST of the polymer sample, the solution is homogeneous and transparent. (b) Above the CST, the aqueous polymer solution exhibits abrupt precipitate formation.

Many theories have been advanced to explain the CST phenomenon and the driving forces participating in the behaviour of thermosensitive polymer solutions [13, 31, 36]. The macroscopically observed aggregation and precipitation is the result of a transition from the

"well-dissolved" coil (below the CST) to the globular state (above the CST) of the individual polymer molecules in solution [37]. As polyNIPAAm has an amphiphilic nature, it seems that hydrophilic bonds (H-bridges) and hydrophobic bonds (between the same and different polymer chains) are at the basic of the CST phenomenon [38]. From a thermodynamic point of view, a system is stable and energetically favoured when $\Delta G < 0$, with $\Delta G = \Delta H - T * \Delta S$, where ΔG: free energy change, ΔH: enthalpy change, T: absolute temperature and ΔS: entropy change. Below the CST, polyNIPAAm molecules exist in solution as extended coils, where hydrophilic interactions (hydrogen bonds) are formed between the side chain amide groups and the surrounding water molecules. This effect produces a $\Delta H < 0$ which dominates the overall free Gibbs energy. However, as the backbone of the polymer and its side chain isopropyl groups (non-polar groups) are hydrophobic, the same process involves a $\Delta S < 0$ because of the formation of icelike cages of water molecules around the solutes [39]. As the temperature increases, the ΔH becomes less negative and the Gibbs free energy (ΔG) increases until a certain temperature (the CST) were the $-T*\Delta S$ component overcomes the ΔH, and ΔG become = 0. If $\Delta G > 0$, the system becomes unstable and a phase separation takes place. This effect is reversible as the polymer can be completely resolubilized when the temperature goes below the CST.

1.4 Construction of the AML

In the literature [40] some important points to consider have been given for the choice of the chemical structure to be chosen for the construction of an AML:

- The AML should not interact strongly with impurities, not even with the AML itself
- The affinity ligand should be accessible for the interaction with the target molecule
- The polymer should have a narrow molecular mass distribution (homogeneous behaviour)
- The phase separation should be complete with a sharp and well characterized phase transition
- The precipitates should be compact but also easily resolubilized
- The process (precipitation, resolubilization) should be reproducible and reversible
- The construction should be economically viable

In order to construct the AML, the carboxylated reactive end-group of the polyNIPAAm was first activated with N-hydroxysuccinimide (NHS), giving a NHS-activated polyNIPAAm. Two different approaches for the construction of the AML were then studied. One was the direct coupling of the polyNIPAAm to the affinity ligand to be used for capturing (figure 1.5). The NHS-activated polyNIPAAm can react with the primary amino groups (N-terminal or lysine amino groups) of the affinity ligand.

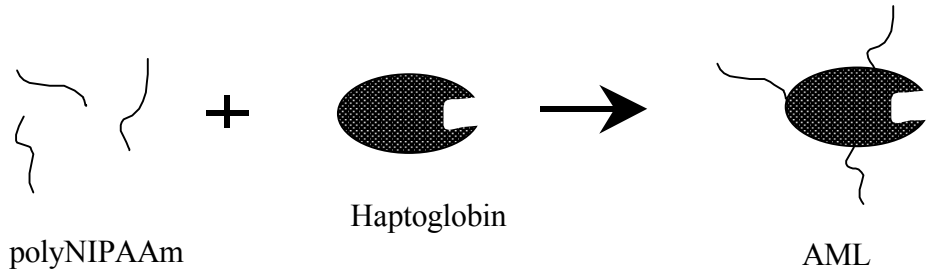


Figure 1.5. Construction of an AML by coupling directly the polyNIPAAm to the affinity ligand. Design of the AML used in chapter 4.

The second approach takes advantages of the high affinity between avidin and biotin (figure 1.6). This interaction is the strongest known non-covalent bond in biochemistry (Ka = 10^{15} M^{-1}, [41]. Up to 4 biotins can be bound per molecule of avidin. To construct the AML, avidin is linked covalently to the polyNIPAAm using the coupling chemistry described above. The ensuing AML is then brought into contact with the biotinylated affinity ligand. AML constructed by this method are bigger as they include the avidin.

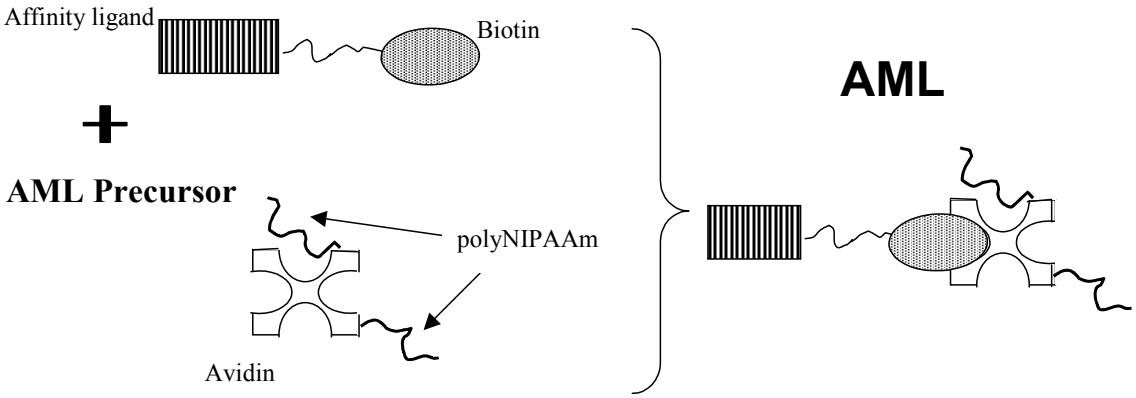


Figure 1.6. Construction of the AML via first the creation of an AML-precursor (Avidin-polyNIPAAm), with subsequent addition of the biotinylated affinity ligand.

1.5 Aim of the thesis

Affinity precipitation is to date not an established downstream processing technique in the biotech industry. The objective of this thesis was to find potential applications for the affinity precipitation by first choosing interesting target molecules and second by comparing the new method with well established affinity bioseparation procedures based on chromatography or magnetic beads. The biomolecules choosen as target products were poly(A) mRNA, scFv antibody phages, hemoglobin and IgG 4E11. To my knowledgement, this is the first time that these biomolecules have been isolated using the affinity precipitation technique.

1.6 References

1. Freitag, R.; Horvath, C. Chromatography in the downstream processing of biotechnological products. Adv. Biochem. Bioeng., **1995**, 53, 17-59.
2. Sadana, A.; Beelaram, A.M. Economics of bioseparation: Some case studies. Bioseparation, **1994**, 4, 221-235.
3. Spalding, B.J. Downstream processing: key to slashing production costs 100 fold. Biotechnology, **1991**, 9, 229-235.
4. Freitag, R.; Costioli, M.; Garret-Flaudy, F. Stimulus-responsive polymers for bioseparation. Chimia, **2001**, 55, 196-200.
5. Garret-Flaudy, F. Thermoresponsive oligomers - solubility and applications for affinity precipitation, **2000**, PhD Thesis EPFL, Lausanne
6. Niederauer, M.Q.; Glatz, C.E. Selective precipitation. Adv. Biochem. Bioeng., **1992**, 47, 160-188.
7. Gupta, M.N.; Mattiasson, B., Affinity precipitation, in Highly selective separation in biotechnology, G. Street, Editor. **1994**, Blackie Academic: London. 7-33.
8. Freitag, R. Reversibly water-soluble affinity macroligands for bioseparation. Cur. Trend Polym. Sci., **1998**, 3, 63-79.
9. Labrou, N.; Clonis, Y.D. The affinity technology in downstream processing. J. Biotech., **1994**, 36, 95-119.
10. Galaev, I.Y.; Mattiasson, B. New methods for affinity purification of proteins. Affinity precipitation: a review. Biochemistry (Moscow), **1997**, 62, 571-577.
11. Hilbrig, F.; Stocker, G.; Schläppi, J.-M.; Kocher, H.; Freitag, R. Utilisation of group specific ligands in the downstream processing of proteins by affinity precipitation. Food Bioprod. Process., **2006**, 84 (C1), 1-9.
12. Galaev, I.Y.; Mattiasson, B. Thermoreactive water-soluble polymers, nonionic surfactants, and hydrogels as reagents in biotechnology. Enzyme Microb Technol, **1993**, 15, 354-366.
13. Schild, H.G. Poly(N-isopropylacrylamide) experiment, theory and application. Prog. Polym. Sci., **1992**, 17, 163-249.
14. Freitag, R.; Baltes, T.; Eggert, M. A comparison of thermoreacive water-soluble poly-N,N-diethylacrylamid prepared by anionic and group transfer polymerization. J. Polym. Sci, **1994**, 32, 3019-3030.
15. Freitag, R.; Garret-Flaudy, F., Affinity macroligands. 1999. US Patent N°: 6,258,275. Application N°: 09/411,148.
16. Ito, S.; Misogushi, K.; Suda, Y. Thermoreversible characteristics of acrylamide derivative polymers. Bull. Res. Inst. Polym. Text., **1984**, 144, 7.
17. Schild, H.G.; Tirrell, D.A. Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions. J. Phys. Chem., **1990**, 94, 4352-4356.
18. Freitag, R.; Garret-Flaudy, F. Salt effects on the thermoprecipitation of poly-(N-isopropylacrylamide) oligomers from aqueous solution. Langmuir, **2002**, 18, 3434-3440.
19. Baltes, T.; Garret-Flaudy, F.; Freitag, R. Investigation of the LCST of polyacrylamides in aqueos solution as a function of molecular parameters and solvent composition. J. Polym. Sci. A, **1999**, 37, 2977-2989.
20. Plate, N.A.; Lebedeva, T.L.; Valuev, L.I. Lower critical solution temperature in aqueous solutions of N-alkyl-substituted polyacrylamides. Polym. J., **1999**, 31, 21-27.
21. Liu, H.Y.; Zhu, X.X. Lower critical solution temperatures of N-substituted acrylamide copolymers in aqueous solutions. Polymer, **1999**, 40, 6985-6990.

22. Freitag, R.; Schumacher, I.; Hilbrig, F. Affinity precipitation an option for early capture in bioprocessing Biotechnol. J., **2007**, 2, 685-690.
23. Nguyen, A.L.; Luong, J.H.T. Synthesis and applications of water-soluble reactive polymers for purification and immobilization of biomolecules. Biotechnol. Bioeng., **1989**, 34, 1186-1190.
24. Kumar, A.; Galaev, I.Y.; Mattiasson, B. Affinity precipitation of alpha-amylase inhibitor from wheat meal by metal chelate affinity binding using cu(II)-loaded copolymers of 1-vinylimidazole with N-isopropylacrylamide. Biotechnol. Bioeng., **1998**, 59, 695-704.
25. Maeda, M.; Nishimura, C.; Inenaga, A.; Takagi, M. Modification of DNA with poly(N-isopropylacrylamide) for thermally induced affinity separation. React. Polym., **1993**, 21, 27-35.
26. Mori, S.; Nakata, Y.; Endo, H. Purification of rabbit C-reactive protein by affinity precipitation with thermosensitive polymer. Protein Expression Purif., **1994**, 5, 153-156.
27. Chen, J.P.; Hoffman, A.S. Polymer-protein conjugates. II. Affinity precipitation separation of human immunogammaglobulin by a poly(N-isopropylacrylamide)-protein A conjugate. Biomaterials, **1990**, 11, 631-634.
28. Costioli, M.D.; Fisch, I.; Garret-Flaudy, F.; Hilbrig, F.; Freitag, R. DNA purification by triple-helix affinity precipitation. Biotechnol. Bioeng., **2003**, 81, 535-545.
29. Chen, G.; Hoffman, A.S. Synthesis of carboxylated poly(NIPAAm) oligomers and its application to form thermoreversible polymer-enzyme conjugates. Polym. Prepr., **1992**, 33, 468-469.
30. Chen, G.; Hoffman, A.S. Preparation and properties of thermoreversible, phase-separating enzyme-oligo(*N*-isopropylacrylamide) conjugates. Bioconjugate Chem., **1993**, 4, 509-514.
31. Heskins, M.; Guillet, J.E. Solution properties of poly(N-isopropylacrylamide). J. Macromol. Sci.-Chem., **1968**, A2, 1441-1455.
32. Hilbrig, F.; Freitag, R. Protein purification for affinity precipitation. J. Chromatogr., B, **2003**, 790, 79-90.
33. Chen, J.P.; Yang, H.J.; Hoffman, A.S. Polymer-protein conjugates. I. Effect of protein conjugation on the cloud point of poly(N-isopropylacrylamide). Biomaterials, **1990**, 11, 625-630.
34. Takei, Y.G.; Matsukata, M.; Aoki, T.; Sanui, K.; Ogata, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. Temperature-responsive bioconjuates. 3. Antibody-poly(N-isopropylacrylamide) conjugates for temperature-modulated precipitations and affinity bioseparations. Bioconjug. Chem., **1994**, 5, 577-582.
35. Costioli, M. Bioconjugates for bioseparation by affinity precipitation, **2004**, PhD Thesis EPFL, Lausanne
36. Zeng, F.; Tong, Z.; Feng, H. N.m.r. investigation of phase separation in poly(N-isopropyl acrylamide) / water solutions. Polymer, **1997**, 38, 5539-5544.
37. Kubota, K.; Fujishige, S.; Ando, I. Single-chain transition of poly(N-isopropylacrylamide) in water. J. Phys. Chem., **1990**, 94, 5154-5158.
38. Graziano, G. On the temperature-induced coil to globule transition of poly-N-isopropylacrylamide in dilute aqueous solutions. Int. J. Biol. Macromol., **2000**, 27, 89-97.
39. Nemethy, G.; Scherega, H.A. Structure of water and hydrophobic bonding in proteins. 1. A model for the thermodynamic properties of liquid water. J. Chem. Phys., **1962**, 36, 3382-3400.

40. Mattiasson, B.; Kumar, A.; Galaev, I.Y. Affinity precipitation of proteins: design criteria for an efficient polymer. J. Mol. Recognit., **1998**, 11, 211-216.
41. Gitlin, G.; Bayer, E.A.; Wilchek, M. Studies on the biotin-binding site of avidin. Biochem. J., **1987**, 242, 923-926.

2. Purification of RT-PCR Competent Poly(A) mRNA from Crude Cell Lysate by Affinity Precipitation

2.1 Introduction

The preparation of messenger RNA (mRNA) from eukaryotic cells and tissues is an important step in many genetic engineering protocols, e.g. for the creation of recombinant production organisms, but also in the analysis of gene structure and regulation. Successful mRNA isolation requires tissue (cell) disruption, denaturation of the nucleoprotein complexes (release of RNA), inactivation of proteins such as RNases and finally separation of the mRNA from other RNA species, as well as residual DNA and protein contaminants. Eukaryotic mRNA has traditionally been purified by a two step-protocol. In the first step the total RNA fraction is captured from the cell lysate / tissue homogenate and thereby separated from cellular proteins, DNA, and most other contaminants. Numerous methods exist for this purpose; all of them disrupt the tissue or cells in the presence of strong protein denaturants to assure the quick inactivation of proteins and especially of RNases. A mixture of 4 M guanidine thiocyanate and β-mercaptoethanol has been found especially effective in this context [1]. The RNA-fraction is then further purified either by several ethanol precipitation steps or by phenol / chloroform extractions followed by isopropanol precipitation steps [2]. Both protocols efficiently remove residual protein contaminants. In addition the RNA-fraction is considerable concentrated by the precipitation. In the second step, the mRNA is selectively purified from the other RNA species present in the RNA-concentrate. This isolation typically relies on the poly(A)-tail, which is present on most mature eukaryotic mRNAs, whereas the other RNA species normally do not carry such a tag. Traditional methods use oligo(dT)- or oligo(dU)-sequences attached to a solid cellulose matrix to selectively capture the poly(A) mRNA from the total RNA preparation.

More recently, we have seen the emergence of methods that avoid the step of total RNA isolation prior to mRNA purification. These methods typically make use of oligo(dT) probes coupled to biotin. The probes are directly mixed into the crude mRNA containing cell lysates for hybridization, thereby taking also advantage of the fact that the hybridization kinetics are faster in solution. Another advantage is that residual particulate matter (cell debris, precipitates) can still be removed by centrifugation at this stage, if soluble probes are used to

anneal the mRNA. Once the biotinylated oligo(dT) probes and the poly(A) mRNA have annealed, the complexes can be specifically captured, e.g. by adding paramagnetic streptavidin-coated beads to the solution. The mRNA-containing complexes will bind via their biotin moieties to these beads and when the beads are captured – typically by using a strong magnet – the complexed mRNA is co-captured. After a series of high stringency washing steps, water is used to release the poly(A) mRNA into solution.

Even if the affinity precipitation has in the past mainly been used for the capture of proteins [3, 4], some applications of the method for polynucleotide isolation have been found. Examples are the capture of double stranded DNA (plasmid DNA) from cell lysates by triple-helix affinity precipitation [5] and the capture of $(dA)_8$ oligonucleotides from aqueous solution presumably also by some form of triple helix interaction using $(dT)_8$-activated polyNIPAAm as AML [6]. The capture of mature eukaryotic poly(A) mRNA by affinity precipitation has never been described, although in this context affinity precipitation could have advantages in terms of cost, handling, and scalability. Moreover, in the case of $(dA)_8$-capture by $(dT)_8$-activated AML, the (dT)-tail was directly linked to the polyNIPAAm via the synthesis of a polymerizable (vinyl) derivative (statistical copolymerization). The approach thus lacks the elegance of using a generic avidin-activated AML to capture a variety of biotin-containing affinity complexes [7].

In this chapter, I show that an avidin-conjugated AML in combination with biotinylated oligo(dT) probes can be used to efficiently purify poly(A) mRNA from crude cell lysates with a yield and a quality that are at least equal to that of a standard approach using streptavidin-activated paramagnetic beads.

2.2 Materials and Methods

2.2.1 Materials

N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), biotin (USP grade), 4-hydroxyazobenzene-2-carboxylic acid (HABA), agarose, buffers salts and organic solvents were from Sigma-Aldrich. Avidin (from egg white, affinity purified) was from KEM-En-TEC Diagnostic (Taastrup, Denmark). Synthetic single-stranded (ss) oligonucleotides, namely ss 5'-ACGGCATCGTCACCAACT-3' (actin exon 1442F), ss 5'-

ACCTAGTCAGAGAGACAAACACCA-3' (actin intron 1788R), ss 5'-AAGCCACCCCACTTCTCTCTAA-3'(Taq actin F), and ss 5'-AATGCTATCACCTCCCCTGTGT-3' (Taq Actin R) were from Microsynth (Balgach, Switzerland). The oligo$(dT)_{16}$ primer for RT-PCR was from Applied Biosystems (Foster City, CA). The ImProm-II reversed transcriptase and the RNasin Plus RNase Inhibitor from Promega were used in the RT-PCR assay. PCR reagents such as buffers, enzymes, RNase-free, sterile, deionized water, and reagents were taken from Promega´s PCR Core system (Promega, Madison, WI) and used according to the manufacturer's protocols. The PolyATtract System 1000 from Promega was used for the capture of poly(A) mRNA by the magnetic beads-based protocol. Millipore water was used to prepare the aqueous solutions unless indicated otherwise.

2.2.2 Analytical methods

The total nucleic acid content of a given sample was estimated spectrophotometrically by UV absorption at 260 nm (spectrophotometer, Lambda 20, Perkin Elmer, Norwalk, CT). Residual dsDNA and protein contamination in the poly(A) mRNA fractions was quantified using the PicoGreen assay and the NanoOrange Protein Quantitation Kit from Molecular Probes (Leiden, The Netherlands), according to the manufacturer's instructions. Samples were diluted in TE buffer (DNA assay) or NanoOrange diluent (protein assay) and prepared in microtubes. The fluorescence was quantified in a microplate fluorometer (Cytofluor 4000, PerSeptive Biosystems, Foster City, CA) using Ex: 485 nm and Em: 530 nm (Pico green) and Ex: 485 nm and Em: 590 nm (NanoOrange). Standard curves were generated using λ-phage DNA (Molecular Probes) and bovine serum albumin (BSA, Sigma-Aldrich). Standards were measured in triplicate, samples in duplicate.

For a more sensitive determination of putative contaminating genomic DNA in the poly(A) mRNA samples, it was attempted to amplify the exon 1 – intron 1 boundary of the β-actin gene (347 bp) by PCR using oligonucleotide primers, 5'-ACGGCATCGTCACCAACT-3' (exon 1442F) and 5'-ACCTAGTCAGAGAGACAAACACCA-3' (intron 1788 R). For the measurement, 1 to 5 µL of sample were pipetted into a PCR reaction solution (final volume 50 µL) containing 1 U of *Taq* DNA polymerase, 1x *Taq* polymerase buffer, 1.5 mM Mg^{2+}, 1 µM of each primer and 200 µM dNTP mix (all Promega). The PCR was run at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec / 60 °C for 1 min / 72 °C for 2 min. A final

extension step was used with 72 °C for 7 min. Amplicons were analyzed by agarose gel electrophoresis (7 cm, 2 % gel in TAE buffer containing 1 µg/mL ethidium bromide, run in horizontal gel electrophoresis unit (Mini-Sub Cell GT system (Bio-Rad, Herkules, CA) coupled to a Bio-Rad PowerPac 300) at 80 V for 45 min using 40 mM Tris-acetate and 1 mM EDTA, pH 8.0 as running buffer). The 1 kb DNA ladder from Invitrogen (Basel, Switzerland) was used for sizing the DNA. 1 µg genomic DNA was used as positive control.

For the specific quantification of the poly(A) mRNA content of the samples, the Poly(A) mRNA Detection System from Promega was used according to the manufacturer's instructions. Briefly, the following volumes multiplied by the number of intended samples were mixed to give the 'master mix': 10.4 µL RNase-free, sterile, deionized water, 2.0 µL 10x DNA polymerase buffer, 0.5 µL sodium pyrophosphate (40 mM), 1.0 µL ADP (20 µM) solution, 0.1 µL nucleoside diphosphate kinase solution, 1.0 µL DNA polymerase solution (Klenow Exonuclease Minus). The master mix was gently mixed and kept on ice until use. The standard curve covering a range of 0 to 500 pg/µL was generated using the 1.2 kb kanamycin mRNA (0.5 µg/µL) included in the kit. Samples were diluted appropriately using RNase-free, sterile, deionized water. For quantification, 10 µL of properly diluted sample, standard or water (for the blank), were mixed with 3 µL of the mRNA-detection oligo mix (1 µg/mL) and 7 µL of RNase-free water and incubated for 15 min at 65 °C for hybridization. After the mixture had cooled to room temperature, 5 µL were added to 15 µL of the master mix, the mixture incubated for 30 min at 37 °C and then placed on ice. Then 100 µL of the rLuciferase / Luciferin Reagent was added to each sample and the light output was measured immediately using a Microplate Luminometer (MicroLumat Plus LB 96V, Berthold Technologies, Vilvoorde, Belgium). Each sample / standard was measured in triplicate.

Sizing of the mRNA, but also a verification of the presence of other RNA species in the final preparation (especially ribosomal RNA, rRNA) was done with the 6000 Pico Assay LabChip Kit from Agilent Technologies (Palo Alto, CA) at the Centre Intégratif de Génomique of the University of Lausanne, Switzerland (Platform 'DNA array facility'). Briefly, 550 µL of the RNA 6000 Pico gel matrix were placed on a spin filter, centrifuged at 1'500 *g* for 10 minutes and divided into 65 µL aliquots. After the addition of 1 µL RNA 6000 Pico dye concentrate, the gel-dye mix was vortexed and centrifuged at 13'000 *g* for 10 min. A RNA 6000 Pico chip was filled with the gel-dye mix using the chip priming station, followed by the addition of conditioning solution and marker. Then 1 µL of sample respectively of diluted (1 : 150), heat-

denatured (70 °C, 2 min) RNA 6000 ladder (Ambion, Huntington, UK) were pipetted into the designated wells, the chip was vortexed for 1 minute and run on an Agilent 2100 Bioanalyzer. RNA-containing samples were heat denatured (70 °C, 2 min) prior to loading on the chip.

To show functionality of the prepared poly(A) mRNA, a fragment of the high-abundance β-actin mRNA (73 bp) was amplified by RT-PCR using a Taq-actin-F primer (5'-AAGCCACCCCACTTCTCTCTAA-3') and a Taq-actin-R primer (5'-AATGCTATCACCTCCCCTGTGT-3'). 2 µL of poly(A) mRNA solution, 1 mM each of dATP, dCTP, dGTP, dTTP, 20 U of ImProm-II reverse transcriptase, 20 U of RNasin Plus RNase Inhibitor and the oligo$(dT)_{16}$ primer (2 µM) were mixed in a final volume of 20 µL. Reverse transcription was performed at 42 °C for 60 min. Then the reaction mixture was heated to 70 °C for 15 min. 1 to 5 µL of the reaction product was subjected to PCR following the same PCR protocol as outlined above. Amplified samples were again analyzed by agarose gel electrophoresis.

Critical solution temperatures (CST) were measured as described by Freitag and Garret-Flaudy [8]. In particular, the optical density of the solution at 600 nm was followed as a function of the temperature (heating rate 1 °C/min). For this the spectrophotometer was equipped with a PTP 1 thermostat and a temperature sensor directly inserted into the reference cell. The CST was approximated as the temperature where the optical density reaches half height.

2.2.3 Synthesis of the AML

PolyNIPAAm with one carboxylic acid end group (AML-precursor) was obtained from the LBCH group (EPFL) and was prepared by telomerization in methanol as described in refs [4, 9-11], i.e. a method that was adapted from Takei et al. [12]. The mass and number average of the molecular weight (M_w and M_n) and the molecular weight distribution (polydispersity P = M_w/M_n) of the polyNIPAAm were calculated from the MALDI-TOF mass spectra (instrument: PerSeptive Biosystems Voyager-DE STR (Atheris Laboratories, Geneva, Switzerland)), using the following formula:

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \qquad M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i}$$

where N_i represents the number of oligomers of a given mass in the sample, while M_i is the mass of a given oligomer species.

Avidin was linked to the AML-precursor by carbodiimide coupling as described by Chen and Hoffmann [13]. For this the NHS-activated polyNIPAAm was obtained as follows: 2 g of polyNIPAAm were dissolved in 30 mL of anhydrous methylene chloride at room temperature. NHS (3.5 mol equivalent) and DCC (1.5 mol equivalent) were added and the mixture was stirred for 3 h. The insoluble precipitate (dicyclohexylisourea) was removed by filtration. The NHS-activated polyNIPAAm was collected and purified by precipitation into anhydrous diethyl ether, filtered, and dried to constant weight. The degree of NHS esterification was evaluated spectrophotometrically (260 nm) by the release of NHS in alkaline solution [14]. For avidin coupling, 35 mg of activated polyNIPAAm (100 mol equiv.) were dissolved in 160 µL of dry DMF (dimethylformamide) and added incrementally at 4 °C to a gently stirred solution of 10 mg avidin in 900 µL of a 0.2 M borate buffer (pH 8.7). The solution was gently stirred overnight at 4 °C. The next morning, 0.3 mL of 0.2 M borate buffer and 0.3 mL of a saturated aqueous ammonium sulfate solution were added. The AML was subjected to a thermo-cycling step (precipitation with ¼ vol $(NH_4)_2SO_4$-saturated solution at 40 °C / centrifugation at 40 °C, 10'000 *g*, 15 min / redissolution in fresh borate buffer at 4 °C). The non-coupled activated polyNIPPAAm was blocked when the AML was redissolved in fresh 0.2 M borate buffer, pH 8.7 at 4 °C containing ethanolamine (final concentration of 0.5 M ethanolamine) followed by a thermo-cycling step. The AML was then redissolved in fresh 0.15 M NaOH solution at 4 °C (hydrolysis of the NHS) followed by a thermo-cycling step. The AML was purified by repeated thermo-cycling steps in fresh nuclease-free water at 4 °C, before transferring it finally into a saline-sodium citrate buffer (0.5x SSC, with 20x SSC: 87.7 g NaCl, 44.1 g sodium citrate in 500 mL RNase-free water, pH adjusted to 7.2 with HCl). The total polyNIPAAm concentration was between 2.5 and 3.0 %. The avidin-content in the AML was determined spectrophotometrically at 280 nm, while the remaining number of biotin binding sites were determined by Green's HABA assay [15] as previously described [4].

2.2.4 Cell culture

The human T leukaemia cell line Jurkat (ATCC TIB152) used as poly(A) mRNA source was cultivated in 75 cm^2 culture flasks in RPMI 1640 (supplemented with 10 % foetal calf serum,

2 mM L-glutamine, 1 mM sodium pyruvate, 1 % non-essential amino-acids, 1 % HEPES, all from Gibco Cell Culture, Invitrogen). Cultures were maintained at 37 °C under 5 % CO_2. The growth medium was changed every other day. Cell density and viability were determined by trypan blue staining and visual inspection in a Neubauer improved hemacytometer (Blau Brand, Wertheim, Germany). For mRNA preparation, cells were centrifuged at 900 rpm (Heraeus Labofuge 400, Kendro, Germany) for 3 min, re-suspended in 25 mL ice cold phosphate buffered saline (PBS) containing 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, and re-centrifuged as above. The supernatant was discarded prior to RNA extraction.

2.2.5 Magnetic Particle Separation of Poly(A) mRNA

The PolyATtract System 1000 from Promega was used according to the manufacturer's instructions for poly(A) mRNA isolation from the Jurkat cells. All experiments were performed under RNase-free conditions. Briefly, 200 µL Extraction Buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.1) supplemented with 5.2 µM β-mercaptoethanol were added to 3 x 10^6 cells (pellet). The mixture was vortexed until complete cell lysis. 400 µL preheated (70 °C) Dilution Buffer (6x SSC, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.25 % SDS) supplemented with 4 µL β-mercaptoethanol were added and mixed thoroughly by inversion. Then, 1 µL biotinylated oligo(dT) probe was added and this mixture was incubated at 70 °C for 5 min. To clean the homogenate of cell debris and precipitated proteins, the total was centrifuged for 10 min at 12'000 *g*. The supernatant was carefully removed with a sterile pipette and added to a tube containing 500 µL washed, streptavidin coated paramagnetic beads (Streptavidin MagneSphere) in saline-sodium citrate buffer (0.5x SSC). After mixing by inversion, the mixture was incubated at room temperature for 2 min. Then, the magnetic particles were captured using the magnetic stand and washed three times with 1 mL 0.5x SSC. To elute the mRNA, 100 µL nuclease-free water was added after the final washing step and the liquid containing the eluted mRNA was transferred to a sterile, RNase-free microcentrifuge tube and stored at –20 °C for further analysis. A summary of the procedure is shown in figure 2.1 (left hand side).

2.2.6 Affinity precipitation of Poly(A) mRNA

Initially these experiments followed the same steps as indicated above for the magnetic beads, see summary of the procedure in figure 2.1. However, instead of the streptavidin-coated particles, the avidin-activated AML was used to capture the poly(A) mRNA as follows. 32.4 mg of non-activated polyNIPAAm (polyNIPAAm lacking the covalently bound avidin) and 3 µL of AML-solution (5 µg avidin/µL) were mixed into 245 µL of 0.5x SSC (final total polyNIPAAm concentration: 3 % w/v) and incubated with 6.5 µL (260 units) of RNasin Plus RNase Inhibitor for 30 min at 4 °C under gently mixing. The supernatant containing the complex of biotinylated oligo(dT) with the poly(A) mRNA was added and the mixture incubated for 5 min at 4 °C (incubation times were not optimized and could probably have been shorter; they were standardized as indicated to assure reproducibility). The poly(A) mRNA / AML-complex was then isolated (thermo-cycling step) by thermoprecipitation at 30 °C (5 min) followed by centrifugation (10'000 *g*, 5 min, 30 °C). For washing (twice), the pellet was redissolved in 1 mL 0.5x SSC at 4 °C, followed by thermoprecipitation at 40 °C (5 min), followed by centrifugation at 10'000 *g* for 5 min at 40 °C and redissolution in fresh 0.5x SSC at 4 °C for the second wash cycle. Finally, the pellet was dissolved in 100 µL of nuclease-free water to detach the poly(A) mRNA and the AML was removed by thermoprecipitation (40 °C, 5 min) followed by centrifugation (10'000 *g*, 40 °C, 5 min). The differences in precipitation / centrifugation temperatures used during mRNA capture compared to the washing and elution steps was due to the different CST of the AML in the corresponding buffers, see also results below.

The supernatant obtained by the outlined procedure was transferred to a sterile, RNase-free microcentrifuge tube and stored at –20 °C for further analysis. Experiments involving the oligo(dT)-activated AML were done as follows. 33.5 mg of non-activated polyNIPAAm were mixed into 245 µL 0.5x SSC and mixed well at 4 °C until fully dissolved. 3 µL of the avidin-activated AML were added together with 6.5 µL (260 units) of RNasin Plus RNase Inhibitor and mixed for 1 h at 4 °C. 245 µL of 0.5x SSC were added together with 1 µL biotinylated oligo(dT) solution. The mixture was incubated for 30 min at 4 °C under gentle agitation to allow the formation of the oligo(dT)-activated AML via the avidin-biotin interaction; afterwards the mRNA-containing sample was added. Subsequent steps were as described above for the avidin-activated AML.

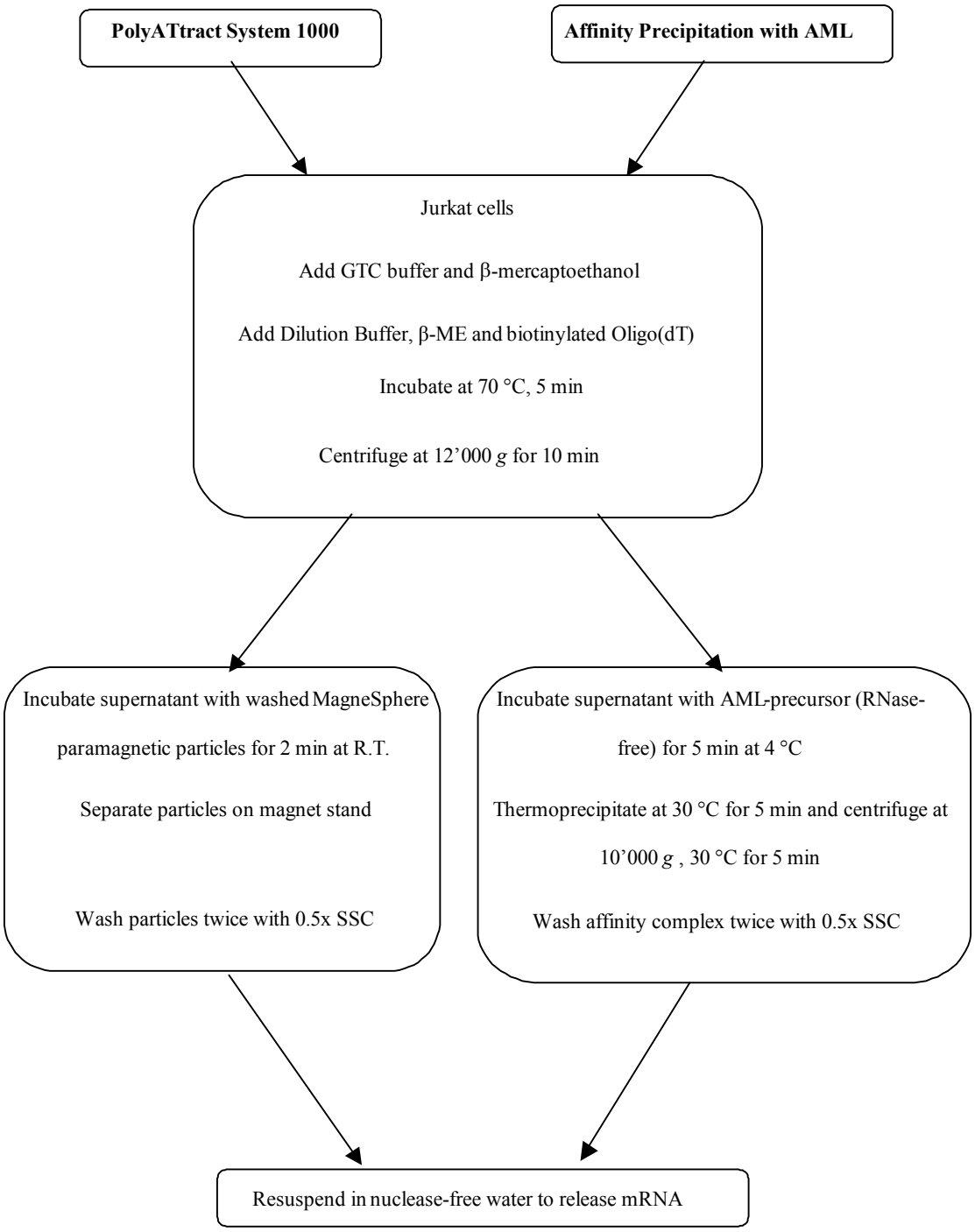


Figure 2.1. Scheme of the poly(A) mRNA isolation procedures. Left hand side: using the PolyATtract System 1000 (Promega). Right hand side: using Affinity Precipitation.

2.3 Results and Discussion

2.3.1 Synthesis and Characterisation of the AML Precursor

According to the analysis by MALDI-TOF, the polyNIPAAm used as AML-precursor for this study had a number average of the molecular weight (M_n) of 2'100 g/mol and a polydispersity of 1.2. Salts and other co-solutes are known to influence the critical solution temperature (CST) of an AML [16]. The attached affinity ligand, on the other hand, typically has little effect, even in the case of fairly voluminous molecules [4, 5]. The solubility-behaviour of polyNIPAAm in a given aqueous solution is therefore a good estimate for the behaviour of a putative polyNIPAAm-based AML. In order to aid process development, the CST of the prepared polyNIPAAm, was therefore recorded for 1 % (w/v) solutions in Millipore water (to mimic the elution conditions in sterile, RNase-free water), in the Extraction buffer / Dilution buffer / β-mercaptoethanol mixture, i.e. conditions approaching those used for cell lysis and RNA hybridization (albeit lacking the expensive biotinylated oligo(dT) probes), and the 0.5x SSC solution used for washing. The results are shown in figure 2.2. According to these results the CST of the polyNIPAAm in water was 32.5 °C, i.e. in the expected range. The CST-value dropped to 31 °C in the 0.5x SSC solution. Given the composition of this solution (4.4 g/L NaCl, 2.2 g/L sodium citrate), this was to be expected. Most noticeable, the CST in the hybridization buffer was as low as 14 °C. Again this value was to be expected given the composition of this particular environment, but the value had to be taken into account when setting up the capture conditions, see below (for a complete treatise on the set-up of an affinity separation, see ref. [7]).

The carboxylic acid end group of the oligomeric polyNIPAAm was subsequently activated by NHS prior to coupling of avidin. A pH of 8.7 (0.2 M borate buffer) was maintained throughout the reaction, as pH-values between 8 and 9 are recommended for ligand conjugation with NHS activated compounds [17]. At lower pH the primary amine groups will be positively charged thus lowering their nucleophilicity, whereas at higher pH the NHS-groups are easily hydrolysed. As verified spectrophotometrically, NHS-activation by the indicated protocol was successful for ca. 90 % of the end groups. This rate was taken into account when calculating the coupling ratios, see below.

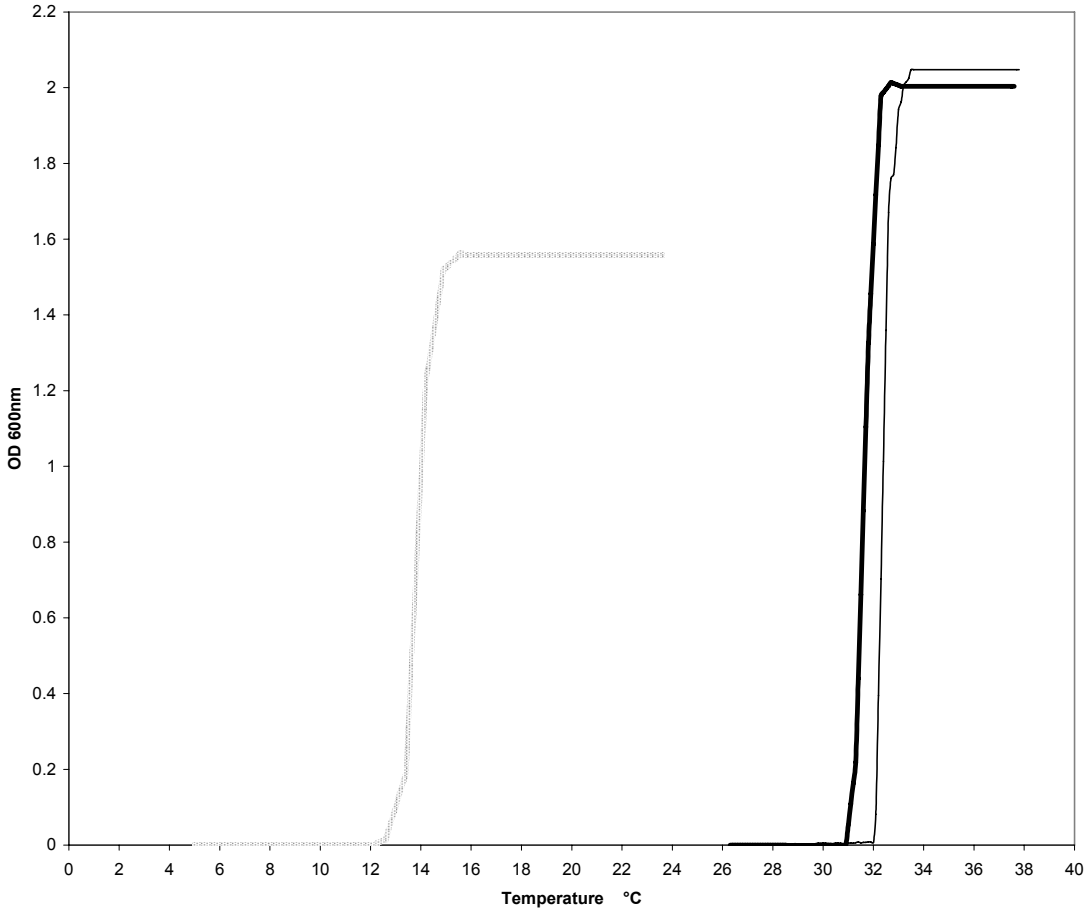


Figure 2.2. Turbidity curves recorded for 1 % (w/v) solution of polyNIPAAm in —— Millipore water, . 0.5x SSC solution, extraction / dilution buffer.

The ratio adjusted between the NHS-activated polyNIPAAm and the affinity ligand may have consequences for both coupling yield (in terms of affinity ligand linkage) and residual binding capacity [5]. Coupling molar ratios of 200 : 1 and 100 : 1 (polyNIPAAm to avidin) were investigated in this study. Considering the fact that each avidin molecule contains 40 primary amino groups, this corresponds to a molar ratio in coupling sites of 5.0 : 1 and 2.5 : 1. In addition, polyNIPAAm concentrations of 5 % (w/v) and 3 % (w/v) were adjusted for the molar ratio of 100 : 1 (only 5 % for 200 : 1). While the coupling yield was highest (97 %) for a molar ratio of 200 : 1 and a polyNIPAAm concentration of 5 %, the average number of residual biotin binding places of the corresponding AML as determined by the HABA assay was only 1.7. By comparison, a molecule of avidin can theoretically bind up to 4 biotin molecules and the HABA assay performed by us with the free avidin prior to coupling indicated an average biotin binding capacity of 3.5 per molecule of avidin. When a molar ratio of 100 : 1 was used with a polyNIPAAm concentration of 3 %, the coupling yield was 75.4 % while the number of biotin binding sites in the AML increased to 2.0. Best results in

terms of combining efficient coupling (yield 85 %) with a high biotin binding capacity (residual binding places: 2.6) were obtained for a molar ratio of 100 : 1 and a polyNIPAAm concentration of 5 % (w/v). The yield of the coupling reaction of the avidin-activated polyNIPAAm with the biotinylated oligo(dT) probe was not determined. However, previous experiments carried out with similar coupling reactions [5, 7] led me to assume a nearly quantitative coupling reaction.

It was subsequently verified that the activation of the polyNIPAAm (NHS, avidin-coupling) did not significantly change the CST for a given buffer system. The oligo(dT)-activated AML was not tested, but showed identical precipitation / redissolution behaviour as the avidin-activated AML in the subsequent affinity precipitations, see below. The most consequential value for the set up of the affinity precipitation of the mRNA was therefore the CST in the RNA-release / hybridization buffer (14 °C). In the subsequent affinity precipitation separations, a temperature of 4 °C was adjusted during hybridization / affinity complex formation, while precipitation / centrifugation was done at 30 °C. During washing steps and for final AML removal after release of the poly(A) mRNA, a precipitation temperature of 40 °C was used to accommodate the higher CST of the AML / affinity complex in these solutions. A value of 4 °C was used in all steps involving AML / complex dissolution, in order to increase the driving force for this reaction.

In addition to the CST-value, salts influence also the morphology of the flocks, which become coarser and precipitation consequently more efficient, when for example ammonium sulfate is added to the mixture. The polymer (AML) concentration, on the other hand, has little influence on the value of the CST in the range of interest (1 to 5 % w/v), but influences the abruptness and completeness of the precipitation [3]. Affinity precipitations in this chapter were all done at final polyNIPAAm concentrations of 2.5 – 3.0 % (w/v) to assure abrupt and quantitative precipitation. In order to reduce the amount of expensive AML used in the experiments to that required for capturing the target molecule, the final polyNIPAAm concentration was provided via the addition of non-activated polyNIPAAm.

Maintenance of an RNase-free environment is prerequisite for RNA-preparation. In order to assure this in the case of affinity precipitation of mRNA, all AML-solutions were treated with 1 Unit/µL of RNasin Plus RNase inhibitor (30 min 4 °C). This treatment had no noticeable

effect on the performance of the AML, while effectively preventing damage of the target RNA-molecules.

2.3.2 Extraction of Poly(A) mRNA from Jurkat Cells

The goal of this investigation was an evaluation of the comparative worth of affinity precipitation in the purification of poly(A) mRNA from cells and tissues. For this purpose, poly(A) mRNA was extracted and purified from Jurkat cells. The basic procedures are outlined in figure 2.1. Following instructions from a commercially available kit (PolyATtract System, Promega), cells were first lysed by chaotropic reagents in the presence of SDS, causing the release of nucleic acids and denaturation and inactivation of proteins including potentially harmful ones such as ribonucleases. The poly(A) mRNA was then hybridized to biotinylated oligo(dT) probes followed by a centrifugation step to remove cellular debris and precipitated proteins. The mRNA hybridized to the oligo(dT) probes should remain in the supernatant after the centrifugation step. The hybridized mRNA was then captured by avidin conjugated polyNIPAAm (affinity precipitation) or by streptavidin coated paramagnetic beads. In order to maximise comparability, aliquots of a given cell lysate were subjected to both affinity precipitation and magnetic bead separation. At least two affinity precipitations and two magnetic bead separations were carried out in parallel to estimate reproducibility of each procedure. Several sets of experiments were included in the investigation.

According to the manufacturer's instruction, the minimal cell number to be used with the magnetic beads kit was 1 x 10^6. Since sample size is often a limiting factor in poly(A) mRNA purification, the corresponding affinity precipitation was also first developed for roughly this cell number, namely 3 x 10^6, taking care to mimic conditions suggested by the kit as closely as possible. Care was also taken to maintain an RNase-free environment throughout, in particular during sample preparation, probe annealing, washes, and mRNA elution. According to the information provided by Promega, 30 pmol of oligo(dT) probe should be used for capture of the total poly(A) mRNA from 1 x 10^6 cells, which in turn is captured with 500 µL of streptavidin-coated paramagnetic beads. From information given by the manufacturer I deduced that this amount of beads represents at least 150 pmol of streptavidin. In the case of the affinity precipitation, AML corresponding to 15 µg of avidin (222 pmol) were used for a similar cell number/oligo(dT) addition. No further optimization of the amount was done, especially since previous experiments using the avidin-biotin system in affinity precipitation

had already demonstrated that such avidin/biotin binding ratios are suitable [5, 7]. In the case of affinity precipitation, the unbound components were removed via two thermoprecipitation-mediated washing steps. Poly(A) mRNA was eluted by the addition of 100 µL nuclease-free water. The final RNA preparation was characterized in terms of total nucleotide content, specific poly(A) mRNA content, as well as residual DNA and protein content. The absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) was used as first crude indication of purity, although other, more specific tests for the presence of certain impurities were to follow. The results of the two separation protocols are summarised in table 2.1.

Table 2.1. Comparison of the purification of poly(A) mRNA using affinity precipitation and paramagnetic beads.

	Affinity precipitation polyNIPAAm-avidin	Affinity precipitation polyNIPAAm-oligo(dT)	Paramagnetic particles
#1 Total polynucleotide content (A_{260})	**580 ng ± 180 ng**		**95 ng ± 15 ng**
from #1, ratio AP/PP [a] (ng/ng)	6.1 ± 2.1		
Purity index (A_{260}/A_{280})	1.80		1.75
#2 Poly(A) mRNA[b]	**440 ng ± 220 ng**	**470 ng ± 130 ng**	**14 ng ± 6 ng**
from #2, ratio AP/PP [a] (ng/ng)	31.4 ± 20.7	33.6 ± 17.1	
Residual dsDNA (two washing steps)	**0.422 ng ± 0.010 ng**	**0.320 ng ± 0.060 ng**	**0.068 ng ± 0.050 ng**
#3 Residual dsDNA/Poly(A)mRNA (ng/ng)	$9.6x10^{-4} \pm 4.8x10^{-4}$	$6.8 x10^{-4} \pm 2.3 x10^{-4}$	$4.9x10^{-3} \pm 4.1x10^{-3}$
from #3, ratio AP/PP [a] (ng/ng)	0.20 ± 0.19	0.14 ± 0.13	
Residual protein (two washing steps)	**560 ng ± 130 ng**	**780 ng ± 105 ng**	**bdl [c]**
Residual protein/ Poly(A)mRNA (ng/ng)	1.27 ± 0.70	1.66 ± 0.51	
Residual dsDNA (three washing steps)	**0.360 ng ± 0.090 ng**		
Residual dsDNA/Poly(A)mRNA (ng/ng)	$8.1x10^{-4} \pm 4.6x10^{-4}$		
Residual protein (three washing steps)	**490 ng ± 90 ng**		
Residual protein/ Poly(A)mRNA (ng/ng)	1.11 ± 0.59		

[a] AP: Affinity Precipitation, PP: Paramagnetic Particles [b] Determined by specific Promega kit. [c] bdl, below the detection limit

According to these results, the magnetic particle-based purification gave 95 ng ± 15 ng polynucleotide (according to the absorbance at 260 nm) with a purity index (A_{260}/A_{280}) of 1.75, while the analogous separation by affinity precipitation yielded 580 ng ± 180 ng of polynucleotides with a purity index (A_{260}/A_{280}) of 1.8 for the same cell lysate. Given the fact that a typical mammalian cell contains roughly 0.5 pg of mRNA per cell (10-30 pg of total RNA / cell, mRNA comprises 1-3 % of total RNA [18]) and that I lysed 3 x 10^6 cells per sample, these yields are extremely low. For the intended application (RT-PCR), these low yields posed no noticeable problem (see results below), however, they may become more significant, e.g. when more complex samples involving RNA from several species (environmental samples) are concerned. Since both protocols the commercial one and the affinity precipitation yielded similar low yields, it is likely that the 'bottleneck' for RNA yield is located in the earlier common steps of the two approaches, i.e. during cell lysis and early RNA release. Moreover, when specifically the poly(A) mRNA content of the preparations was determined via the poly(A) mRNA detection system from Promega, which is designed for the sensitive and specific detection of total polyadenylated messenger RNA in RNA preparations, the amount of poly(A) mRNA in the 580 ng obtained by affinity precipitation was 440 ng while this value was as low as 14 ng in case of the 95 ng prepared by extraction using magnetic beads.

The residual contamination by dsDNA per ng of polynucleotide was in the same order of magnitude in the two cases, while the relative contamination of the prepared mRNA by dsDNA was somewhat lower in case of the affinity precipitation. A contamination by proteins was still detectable in case of the mRNA prepared by affinity precipitation, while this contamination was below the detection limit in case of the mRNA prepared using magnetic particles. Given the lower absolute amount of polynucleotide prepared in this case, such a difference does not necessarily represent a real difference in purification efficiency. Indeed, for the intended application of the purified mRNA, i.e. amplification of certain sequences by RT-PCR this residual protein contamination made no apparent difference, see results below. For mRNA prepared by affinity precipitation, both the contamination by dsDNA and proteins can be further reduced by adding a third washing step to the protocol, see table 2.1. The stability of the complex under binding conditions is high enough to allow repeated dissolution reprecipitation cycles. Since the complex is fully dissolved during washing, these steps are highly efficient for removing residual impurities.

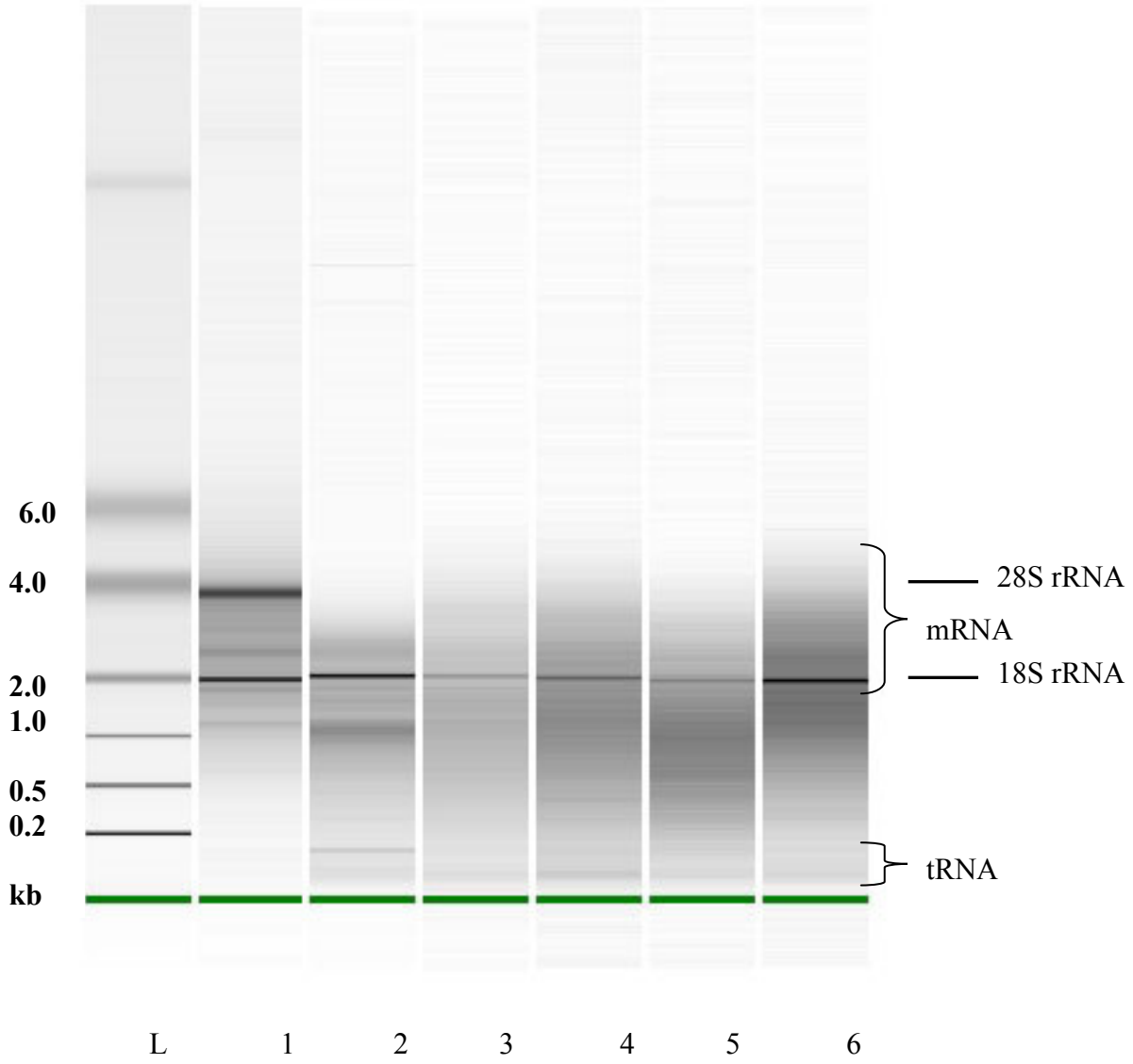


Figure 2.3. Analysis by chip electrophoresis using the RNA 6000 Pico Assay LabChip Kit and the Agilent 2100 Bioanalyzer (Agilent) of poly(A) mRNA isolated from Jurkat cells. L: RNA 6000 Ladder (six RNA transcripts 0.2, 0.5, 1.0, 2.0, 4.0 and 6.0 kb, the 0.2 kb band is at a concentration of 10 ng/µL (A_{260})); lane 1: poly(A) mRNA purified by the PolyATtract System 1000; lane 2: poly(A) mRNA purified by affinity precipitation as summarized in figure 2.1, lanes 3 and 4: poly(A) mRNA purified by affinity precipitation using oligo(dT)-activated AML, lanes 5 and 6: poly(A) mRNA purified by affinity precipitation using a third washing step.

In order to further analyse the composition of the poly(A) mRNA samples obtained by both procedures, aliquots were subjected to gel electrophoresis using the Agilent Bioanalyzer 2100. As shown in figure 2.3, the sample purified with the magnetic beads still contained significant amounts of both the 18S and the 28S rRNAs. Traces of tRNA are also present. In the mRNA prepared by affinity precipitation, only the band for the 18S rRNA is observable in the gel. Some contamination of the purified mRNA with rRNA is to be expected given the

relative abundance of the two types in the cell (rRNA up to 80 %, mRNA 1-3 % of the total RNA in the cell [18]). Moreover, rRNA has been known to co-purify with mRNA due to non-specific interaction e.g. with the oligo(dT) matrix or due to specific hybridization of rRNA and mRNA sequences [19]. The ability of the affinity precipitation to reach a better separation between the two RNA species may be due to the inertness of the carrier (polyNIPAAm) but also to the fact that original capture of the biotinylated mRNA and the individual washing steps take place in homogeneous solution. Also seen in the gel (figure 2.3) is the fact that the addition of a third washing step to the affinity protocol helps to further reduce the contamination in the mRNA preparation by other RNA species.

To test the quality of the poly(A) mRNA on a functional basis, the mRNA extracted from the Jurkat cells by affinity precipitation as well as by magnetic beads was used in a standard reverse transcriptase amplification assay. In particular, the mRNA for the human cytoplasmic β-actin gene was targeted by the RT-PCR. A primer set located in exon 5 (Taq-Actin-F / Taq-Actin-R, amplicon 73 bp) was used. cDNA was generated from poly(A) mRNA using the Improm-II™ reverse transcriptase (Promega). The cDNA was amplified with Taq DNA polymerase (Promega). In addition, to estimate the presence of putative traces of genomic DNA in the mRNA preparation, a 347bp fragment of the β-actin gene was targeted by PCR (primers located at the boundary of exon 1 and intron 1, exon1442F / intron1788R). 1 µg of human genomic DNA was used as positive control. The results are summarized in figure 2.4.

For both mRNA preparations the β-actin gene fragment was amplified by RT-PCR, as evidenced by a band in the gel in the expected position (lane 3 for mRNA prepared by magnetic beads, lane 5 for mRNA prepared by affinity precipitation). While this is a strong indication for the successful isolation of the corresponding mRNA (fragment), it cannot be excluded that the amplicon stems from a residual DNA contamination (amplified by the PCR). However, if no reverse transcription was performed prior to PCR (lane 12), no amplification of the gene sequence was observed, i.e. the band observed in lane 5 must indeed stem from successfully isolated RNA. This absence of genomic DNA is further corroborated by the fact that it was not possible to amplify the second target, i.e. the 347bp-fragment spanning the exon-intron boundary of the β-actin gene by (RT-)PCR of the mRNA preparations. The corresponding lanes 2 (magnetic particles) and 4 (affinity precipitation) show no evidence of such amplicons.

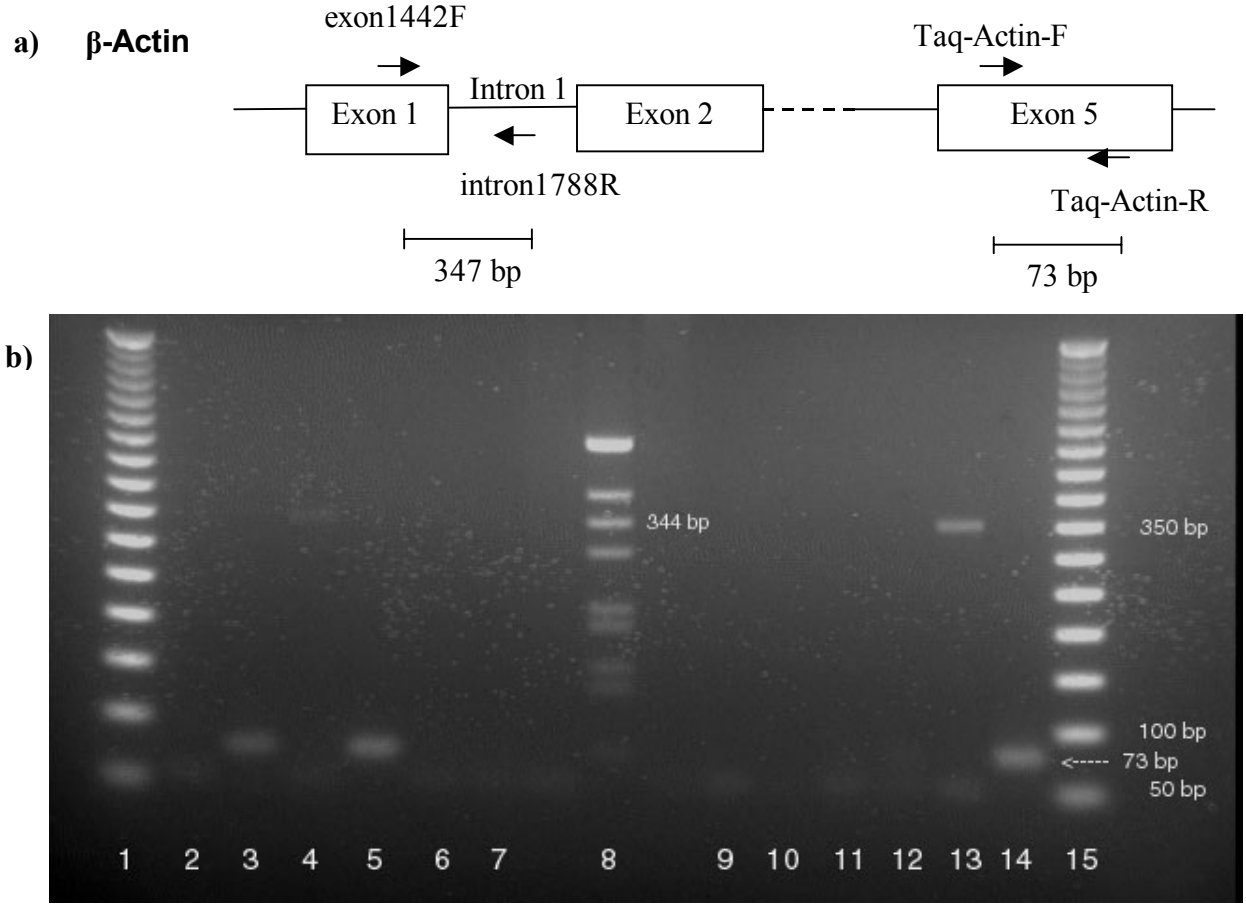


Figure 2.4. a) β-actin gene and primers used for RT-PCR analysis. b) 2.0 % agarose gel run in 1x TAE buffer with 0.1 % EtBr of the amplicons generated from the poly(A) mRNA gene extracted from Jurkat cells. The targets DNA are the 73 bp fragment corresponding to the human cytoplasmatic β-actin gene and a 347 bp fragment corresponding to an exon-intron overlap (indication of a contamination by genomic DNA) of this gene. The positive control for the PCR used 1 µg genomic DNA. Lanes 1 and 15: PCR marker, 50 bp DNA step ladder, (Promega). Lane 2: mRNA isolated with magnetic beads, primers exon1442F / intron1788R. Lane 3: mRNA isolated with magnetic beads, primers Taq-Actin-F / Taq-Actin-R. Lane 4: mRNA isolated by affinity precipitation, primers exon1442F / intron1788R. Lane 5: mRNA isolated by affinity precipitation, primers Taq-Actin-F / Taq-Actin-R. Lane 6: negative control (water, no mRNA) with primers exon1442F / intron1788R. Lane 7: negative control (water, no mRNA) with primers Taq-Actin-F / Taq-Actin-R. Lane 8: PCR marker, 1 kb DNA Ladder (Invitrogen). Lane 9: no genomic DNA (water) with primers exon1442F / intron1788R. Lane 10: no genomic DNA (water) with primers Taq-Actin-F / Taq-Actin-R. Lane 11: without reverse transcriptase, isolation of mRNA with magnetic beads, primers exon1442F / intron1788R. Lane 12: without reverse transcriptase, isolation of mRNA by affinity precipitation, primers Taq-Actin-F / Taq-Actin-R. Lane 13: genomic DNA with primers exon1442F / intron1788R. Lane 14: genomic DNA with primers Taq-Actin-F / Taq-Actin-R.

2.4 Conclusions

The use of magnetic beads has found widespread use for the selective and highly efficient co-capture and concentration of biological molecules. However, the use of suitably activated stimuli-responsive polymers, like polyNIPAAm, could have advantages over the use of beads / magnets under certain circumstances [3, 20]. Poly(A) mRNA can be selectively captured and purified from crude cell lysates by affinity precipitation. Affinity precipitation is thus a viable alternative to magnetic beads-based separation of poly(A) mRNA. In my hands the precipitation was easier to handle and more reproducible than the magnetic particle-based separation. Putative problems with RNases could be prevented by a suitable treatment of the AML-preparation. Compared to the standard technique (extraction via magnetic beads), better yields and higher quality were obtained. The produced poly(A) mRNA proved to be an excellent target for RT-PCR amplification.

Scale up of affinity precipitation presents no problem [3], while this is increasingly difficult with magnetic beads-based separation schemes. No dedicated equipment other than a centrifuge and hot and cold water (thermostated water bath) are needed for affinity precipitation. Moreover, the particular requirements of magnetic beads-based separations make a two step protocol unavoidable, where first the mRNA is hybridized in solution with the oligo(dT)-probes, which – after removal of debris and precipitates by centrifugation – are then in a second step captured by the magnetic beads. In affinity precipitation it is possible to create a fully functional, i.e. oligo(dT)-activated AML either by the avidin-biotin approach or by directly linking the oligo(dT) to the polyNIPAAm [4, 21]. These AML can then be used directly to capture the mRNA from cell lysates. Removal of debris and (protein) precipitates is possible in the presence of the AML, as long as the temperature of the hybridization mixture is below the CST. When the direct coupling approach (oligo(dT) AML) was used in an otherwise similar separation protocol, the yield of poly(A) mRNA was slightly higher than for the avidin-activated AML, table 2.1, while the procedure became more reproducible than for the two step protocol (i.e. hybridization with biotinylated oligo(dT) followed by affinity precipitation using avidin-activated AML). The residual contamination by dsDNA and proteins was similar. Compared to magnetic beads, the costs of the polyNIPAAm based AML are low and could presumably still be lowered by linking the oligo(dT) directly to the polymer instead of using the avidin-biotin interaction.

2.5 References

1. Chirgwin, J.M.; Przybyla, A.E.; Macdonald, R.J.; Rutter, W.J. Isolation of Biologically-Active Ribonucleic-Acid from Sources Enriched in Ribonuclease. Biochemistry, **1979**, 18, 5294-5299.
2. Chomczynski, P.; Sacchi, N. Single-Step Method of Rna Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. Analytical Biochemistry, **1987**, 162, 156-159.
3. Hilbrig, F.; Freitag, R. Protein purification for affinity precipitation. J. Chromatogr., B, **2003**, 790, 79-90.
4. Hilbrig, F.; Stocker, G.; Schläppi, J.-M.; Kocher, H.; Freitag, R. Utilisation of group specific ligands in the downstream processing of proteins by affinity precipitation. Food Bioprod. Process., **2006**, 84 (C1), 1-9.
5. Costioli, M.D.; Fisch, I.; Garret-Flaudy, F.; Hilbrig, F.; Freitag, R. DNA purification by triple-helix affinity precipitation. Biotechnol. Bioeng., **2003**, 81, 535-545.
6. Mori, T.; Umeno, D.; Maeda, M. Sequence-specific affinity precipitation of oligonucleotide using poly(N-isopropylacrylamide)-oligonucleotide conjugate. Biotechnol. Bioeng., **2001**, 72, 261-268.
7. Freitag, R.; Hilbrig, F., Use of the avidin (imino)biotin system as general approach to affinity precipitation, in Avidin-Biotin Interactions, Methods and Applications, R.J. McMahon, Editor. **in press**, Methods in Molecular Biology, 418, The Humana Press Inc.: New Jersey.
8. Freitag, R.; Garret-Flaudy, F. Salt effects on the thermoprecipitation of poly-(N-isopropylacrylamide) oligomers from aqueous solution. Langmuir, **2002**, 18, 3434-3440.
9. Garret-Flaudy, F. Thermoresponsive oligomers - solubility and applications for affinity precipitation, **2000**, PhD Thesis EPFL, Lausanne
10. Costioli, M. Bioconjugates for bioseparation by affinity precipitation, **2004**, PhD Thesis EPFL, Lausanne
11. Baltes, T.; Garret-Flaudy, F.; Freitag, R. Investigation of the LCST of polyacrylamides in aqueos solution as a function of molecular parameters and solvent composition. J. Polym. Sci. A, **1999**, 37, 2977-2989.
12. Takei, Y.G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparation. Bioconjugate Chem., **1993**, 4, 341-346.
13. Chen, G.; Hoffman, A.S. Preparation and properties of thermoreversible, phase-separating enzyme-oligo(*N*-isopropylacrylamide) conjugates. Bioconjugate Chem., **1993**, 4, 509-514.
14. Miron, T.; Wilchek, M. A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters. Anal. Biochem., **1982**, 126, 433-435.
15. Green, N.M. Specrophotometric determination of avidin and biotin. Methods Enzymol., **1970**, 18A, 418-424.
16. Garret-Flaudy, F.; Freitag, R. Use of the avidin (imino)biotin system as a general approach to affinity precipitation. Biotechnol. Bioeng., **2001**, 71, 223-234.
17. Ding, Z.L.; Chen, G.H.; Hoffman, A.S. Synthesis and purification of thermally-sensitive oligomer-enzyme conjugates of poly(N-isopropylacrylamide)-trypsin. Bioconjug. Chem., **1996**, 7, 121-125.
18. *RNA preparation, SuperArray*. Available from: http://www.superarray.com/newsletter/RNA.html.

19. *Ambion Technotes 8(4): Big advances in mRNA purification*. Available from: www.ambion.com.
20. Galaev, I.Y.; Mattiasson, B. Thermoreactive water-soluble polymers, nonionic surfactants, and hydrogels as reagents in biotechnology. Enzyme Microb. Techno., **1993**, 15, 354-366.
21. Costioli, M.D.; Freitag, R.; Hilbrig, F. Strategies for the efficient synthesis of stimuli-responsive ss-DNA bioconjugates for capture and transfection of plasmid DNA. Reactive & Functional Polymers, **2005**, 65, 351-365.

3. Screening of a synthetic human antibody phage display library against the MUC-1 surface antigen using smart bioconjugates

3.1 Introduction

Recombinant antibodies have become important and powerful tools in the fight against cancer. Typically such antibodies are directed against surface structures that are found on cancer cells but not – or much less numerously – on normal body cells. Our group has for a while been interested in the Mucin-1 protein, MUC-1, a highly glycosylated transmembrane protein ubiquitously present in many human tissues [1]. The extracellular domain of MUC-1 is made up of a variable number of tandem repeats of 20 amino acids in length (SAPDTRPAPGSTAPPAHGVT) [2]. In contrast to the MUC-1 expressed by normal cells, MUC-1 in malignant tumour cells shows both an increase in expression and a tendency for under-glycosylation, as e.g. observed in more than 90 % of all breast cancer cells [3, 4]. As a consequence, structures not found on normal cells are exposed on such tumour cells, which can be potential targets for immunotherapy [5].

A variety of methods for the production of specific antibodies against a given antigen have been described. In this context phage display [6] has been described as the methods of choice for the quick screening of large libraries of recombinant antibody fragments against cell surface antigens [7-10]. Antibody phage display libraries typically contain a billion different antibodies, a number comparable to that in the human immune system, and can be used to select antibodies against any given antigen, including cell surface structures like MUC-1 [11].

The antibody phage library used by us, is a modified version of the synthetic antibody library described by Pini et al. [12], based on the variable regions of heavy (V_H) and light (V_L) chains of human antibodies, which are randomly combined and linked together by a polypeptide linker to form so-called single-chain fragments (scFv). Altogether the library comprises around 10^{13} clones (10 variable positions, 20 amino acids possible for each). These scFv are fused to a minor coat protein of bacteriophage M13, pIII, resulting in phages displaying the corresponding antibody fragments on their cell surface. Phages exposing a potential antibody fragment are selected by panning the library for several rounds on the antigen [13]. Various

methods have been proposed of how best expose the antigen to the phage library. Selection on living cells has been suggested [11], but requires delicate manipulation of the cells. More robust alternatives include selection on antigen-coated immuno tubes [14] and the interaction with the biotinylated antigen in solution followed by capture of the associated complex using streptavidin-conjugated paramagnetic beads [15]. Immuno tubes sometimes have difficulties with the accessibility of the antigen, especially in case of fairly small peptide antigens. Magnetic beads work well, but require dedicated equipment. In addition, the corresponding methods are difficult to scale up or to adapt to high throughput screening. Again, stimuli-responsive bioconjugates may present an interesting option for such bioseparations.

In this chapter the potential of stimuli-responsive agents as tools in the screening of an antibody phage library is investigated, taking the MUC-1 surface antigen as example. As far as I know, this is the first time a structure as large as a viral particle has been pulled down by a stimuli-responsive molecule.

3.2 Materials and Methods

3.2.1 Materials

Unless stated otherwise, chemicals and solvents were from established suppliers such as Sigma-Aldrich and used as obtained. Millipore water was used to prepare the aqueous solutions unless indicated otherwise. Stimuli-responsive polymers were as described previously in chapter 2, and obtained from polyTag Technology, Männedorf, Switzerland (see also ref [16, 17]). The AML-precursor was obtained after bioconjugation of avidin with the stimuli-responsive polymers as described in chapter 2 (see also ref [16]). N-α-Fmoc protected amino acids, preloaded cysteine (trityl) 2-chlorotrityl resin, and the activator 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) were from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Synthetic single-stranded (ss) oligonucleotides, namely ss 5'-GAATTTTCTGTATGAGG-3' (fdseq1), ss 5'-CAGGAAACAGCTATGAC-3' (LMB3), and ss 5'-TACTACGCAGACTCCGTGAAG-3' (DP47CDR2back) were from Microsynth (Balgach, Switzerland). PCR reagents such as Tfl (polymerase), buffers, PCR nucleotide mix, Mg^{2+} solution and nuclease-free, sterile, deionized water were from Promega (Madison, WI) and used according to the manufacturer's protocols. Streptavidin magnetic beads were taken from Promega´s PolyATtract System

1000. N-Iodoacetyl-N-biotinylhexylene diamine (Iodoacetyl-Biotin) was from Uptima, Interchim (Montluçon Cedex, France). D-glucose was from Acros Organics (Geel, Belgium). Lennox L Broth Base (LB medium), UltraPure™ Agarose, UltraPure™ 10X TAE buffer, and ammonium sulfate were from Invitrogen (Basel, Switzerland). Stock solutions of antibiotics dissolved in water were sterilized by filtration through a 0.22 µm filter. All biological buffers and media were autoclaved. Minimal plates were prepared by autoclaving 1 litre of a solution containing 60.4 mmol K_2HPO_4, 33.1 mmol KH_2PO_4, 7.6 mmol $(NH_4)_2SO_4$, 1.7 mmol Na_3citrate, and 15 g agar in water. The medium was cooled to 55-58 °C, 1 mL of a 1 M $MgSO_4$ solution (autoclaved), 10 mL of a 20 % (w/v) glucose solution (autoclaved) and 0.5 mL of a 1 % (w/v) thiamine solution (sterilized by passing through a 0.22 µm filter) were added. Plates were stored at 4 °C. TYE-GLU-AMP (or TYE-GLU-KAN) plates were prepared by autoclaving 1 litre of TYE medium (8 g tryptone, 5 g yeast extract and 5 g NaCl in 1 litre of water) with 15 g agar. The medium was cooled to 55-58 °C, glucose was added to a final concentration of 1 % and 1 mL of a 100 mg / mL solution of ampicillin (respectively 1 mL of a 25 mg / mL solution of kanamycin) were added. TYE-GLU-AMP and TYE-GLU-KAN plates were stored at 4 °C.

3.2.2 Analytical methods

The amount of purified phagemid DNA was quantified via the optical density at 260 nm (OD_{260}), plasmid purity was evaluated via the OD_{260}/OD_{280} ratio using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The masses of the peptide were determined by electrospray ionization mass spectrometry with time-of-flight detection on a LCT mass spectrometer from Micromass (Manchester, UK). For the determination of the critical solution temperatures (CST), the optical density of the solution at 600 nm (OD_{600}) was followed as a function of the temperature (heating rate 1 °C/min).

For the phage-ELISA, microtiter plates (Nunc-Immuno™ plates, Maxisorp; Nunc International) were coated with 100 µL per well of a 250 µg/mL MUC-1 (for peptide synthesis see below) solution in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02 % NaN_3, pH 9.6) and incubated overnight at 4 °C. The next morning the plates were washed 3 times with PBS (8.2 mmol Na_2HPO_4, 1.5 mmol KH_2PO_4, 137 mmol NaCl, and 2.7 mmol KCl in 1 L water, pH 7.4, autoclaved) containing an additional 0.1 % Tween-20 (PBS-Tween), then 3 times with PBS, and blocked with 200 µL per well of blocking buffer (1 % (w/v)

Casein, 0.05 % (w/v) Tween-20 in PBS) for 2 h at room temperature under agitation (600 rpm, Titramax 101 Shaker, Heidolph Instruments, Schwabach, Germany). The plates were washed as described above. 80 µL from the polyethylene glycol-precipitated polyclonal phage antibodies ('PoPhAbs', see protocol 'rescue of selected phages') or from a culture supernatant of superinfected *E. coli* containing monoclonal phage antibodies ('MoPhAbs', see protocol 'rescue of phages for monoclonal phage ELISA') were added together with 20 µL of a solution containing 5 % Casein and 0.25 % Tween-20 in PBS and incubated for 2 h at room temperature and 600 rpm. PBS or LB medium served as negative control, while the full library was the positive control. The plates were washed and 100 µL of a solution (stock solution diluted 5-fold) of HRP/Anti-M13 monoclonal conjugate antibody (Amersham Pharmacia Biotech, Buckinghamshire, England) in blocking buffer were added per well and incubated 1 h at room temperature and 600 rpm prior to washing. 100 µL per well of ABTS (substrate) were added and the plate incubated for 15 to 60 min at room temperature and 600 rpm for development. The absorbance was measured at 405 nm using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A)

3.2.3 Peptide synthesis and biotinylation

The MUC-1 peptide sequence (NH_2-SAPDTRPAPGSTAPPAHGVT-COOH) was synthesized in modified form by adding a GGGGG-C sequence to the C-terminus. Synthesis was performed on an AMS 422 peptide synthesizer from ABIMED (Langenfeld, Germany). The synthesizer was charged with preloaded H-Cys(Trt)-2ClTrt resin (0.57 mmol/g). A 4-fold excess of N-α-Fmoc protected amino acid to peptide chain was used in each step and the Fmoc protection group was removed after each cycle with 1.5 mL of 20 % (v/v) piperidine in DMF. The amino acids were activated *in situ* with 350 µL of a 0.57 M solution of HBTU in DMF and 100 µL of a 44 % (4 M) solution of NMM (N-methylmorpholine) in DMF. Some droplets (corresponding to ca. 50-100 µL) of dichloromethane were added during the coupling. At the end of the synthesis, the peptide was cleaved from the resin and the side chain deprotected by a 2 h incubation (with stirring) at ambient temperature in 2 mL of a 'cleavage cocktail' (94 % TFA (trifluoroacetic acid), 2.5 % water, 2.5 % EDT (ethanedithiol), 1 % TES (triethylsilane)). The peptide solution was separated from the resin by filtration and recovered in a 15 mL polypropylene centrifugation tube. To precipitate the peptide, 10 mL of frozen ether were added and the solution incubated for a minimum of 15 min at –80 °C, followed by centrifugation at 2'000 *g* (10 min, 4 °C). The pellet was resuspended in 1 mL 50

% acetic acid, and then 6 mL of ultra pure water were added. The tube was frozen and the peptide was lyophilized (Lyophilisator Christ Alpha 1-2, Martin Christ GmbH, Osterode, Germany). The raw peptide was dissolved in eluent A and purified by RPC (reversed phase chromatography) on a 10 x 100 mm (5 µm particles) Xterra PrepMS C18 column (Waters, Switzerland). Eluent A was water containing 0.1% TFA. Eluent B was acetonitrile containing 0.1% TFA. Elution was in a linear gradient from 20 to 90 % B in 30 min at a flow rate of 2.5 mL/min.

For biotinylation, a solution of the peptide at a concentration corresponding to 5.3 mg/mL (2.35 mM) of free, i.e. reduced, sulfhydryl groups was prepared in biotinylation buffer (0.1 M ammonium bicarbonate, 5 mM EDTA, pH 8.5). The iodoacetyl-biotin was dissolved at a concentration of 50 mg/mL (97.9 mM) in DMSO. 250 µL of this iodoacetyl solution were added to 3.75 mL of peptide solution. The two solutions were mixed and incubated for 2 h protected from light at ambient temperature. Afterwards the reaction was blocked by adding 100 µL of a 120 mg/mL L-cysteine·HCl solution and incubated for 30 min at ambient temperature. The biotinylated peptide was further purified by gelfiltration (column: Superdex TM peptide 10/30) using an FPLC (column and instrument: Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with the mobile phase (0.1 M ammonium bicarbonate, pH 8) and the peptide solution (sample preparation: centrifugation at 10'000 *g*, 10 min, ambient temperature) was loaded at a flow rate of 0.8 mL/min. Development was done at the same flow rate and 3 to 4 mL fractions were collected. The purified peptide was lyophilized.

3.2.4 Bacteria culture

E. coli strain TG1 K12, Δ(lac-pro), supE, thi, hsdD5/F' traD36, $proA^+$ B^+, $lacI^q$, lacZΔM15 was used as host for the bacteriophages. Exponentially growing *E. coli* cultures, which were necessary for phage titration and for the phage rescue after selection, were obtained as follows: Colonies were taken from a TG1 minimal plate and added to 3 mL LB-GLU medium (1 % glucose in LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl in 1 L water)) in a 10 mL sterile tube. The culture was incubated overnight at 37 °C and 200 rpm (Multitron incubator shaker, type AJ 115, HT Infors, Bottmingen, Switzerland). 1 mL of this overnight culture was added to 50 mL of LB-GLU in a 250 mL sterile polycarbonate Erlenmeyer (Corning Inc, USA) prior to incubation at 37 °C and 120 rpm. An OD_{600} between 0.4 and 0.5

guaranteed that the bacteria growth was in the exponential phase. If necessary, cultures were kept on ice for a maximum of 30 min before infection.

3.2.5 Phage library and handling

3.2.5.1 ETH-2 library

The ETH-2 library used in this study is a phage display library of human recombinant scFv antibodies [18]. The library was supplied by the Swiss Federal Institute of Technology in Zurich (Prof. Dario Neri). It consists of four sub-libraries, prepared in identical fashion but on different days: ETH-1, ETH-1a, ETH-1b, ETH-1c. Three antibody germ lines were used (DP-47 for the V_H, DPK-22 and DPL-16 for the V_L) for the generation of the library. To produce a large repertoire, a short variable complementary-determining region 3 (CDR3) was appended to them. At position 95 of the V_H a random loop of 4 to 6 amino acids was introduced (…C^{92} A K $(X)_{4\text{-}6}$ F D Y…). Diversity on the light chain sequences was introduced by randomizing amino acids at six different locations. The phagemid vector, pDN332, derived from the phagemid pHEN1 [19] was used for cloning of the library.

3.2.5.2 Initial rescue of the phage library (original stock)

50 µL bacterial library stock were inoculated into 50 mL of LB-GLU-AMP medium (LB containing 1 % Glucose and 100 µg/mL ampicillin) and incubated at 37 °C and 120 rpm until the OD_{600} reached a value between 0.4 and 0.5. This culture was infected with helper phage (see below for preparation) by mixing them in the ratio of 1 : 20 (bacterial cells to helper phage particles). The helper phages provide the genes, which are essential for phage replication and assembly, as a phagemid alone cannot produce infective phage particles. The mixture was incubated at 37 °C for 30 min. The infected bacteria were spun down at 3'300 *g* for 10 min at room temperature. The pellet was gently resuspended in 500 mL LB-AMP-KAN (LB containing 100 µg/mL ampicillin and 25 µg/mL kanamycin) followed by overnight incubation at 30 °C and 120 rpm. Phages were recovered (phage recovery protocol) and titrated (phage titration protocol) as described below.

3.2.5.3 Preparation of helper phage stock

Helper phages were produced from a sample provided by Dr. Robert Driscoll, Research Associate at the ISREC in Lausanne, Switzerland. In the first step colonies of *E. coli* infected

with helper phage were prepared on TYE-GLU-KAN plates (following the titration phage protocol given below). From these plates a small plaque was picked into 3 to 4 mL of an exponentially growing culture and incubated for 2 h at 37 °C and 120 rpm. This culture was inoculated into 500 mL LB-GLU medium in a 2 L flask and grown for 1 h. Then kanamycin to a final concentration of 50 to 70 µg/mL was added followed by overnight incubation (37 °C, 120 rpm). Helper phages were recovered (phage recovery protocol) and titrated (phage titration protocol) as described below.

3.2.5.4 Recovery of phages (phage library or helper phage)

For phage recovery the bacteria were removed from solution by centrifugation (3'500 *g*, 4 °C, 1 h) and to 500 mL of the phage-containing supernatant 125 mL of a solution containing 20 % PEG 6000 and 2.5 M NaCl in water were added. After mixing, the solution was incubated for 1 h at 4 °C. Precipitated phages were recovered by centrifugation (3'500 *g*, 4 °C, 1 h) and the pellet was resuspended in 3 mL PBS (for phage library), or in 2 ml LB medium (for helper phage) and filtered through a 0.45 µm filter (Nalgene, Nunc International, Rochester, NY).

3.2.5.5 Phage / helper phage titration

Phage titration is a step necessary to determine the concentration of the phage library stock (or of the helper phage stock, or of the selected phages after each round of panning) in order to determine amplification and/or selection efficiency. For this purpose, undiluted phage stocks were diluted 1 : 10^6 in LB medium. 10 µL of this diluted preparation was used to infect 1 mL of an *E. coli* culture in the exponential phase (incubation for 30 min at 37 °C, no agitation). A serial dilution (10 x) was done and 100 µL of each dilution were plated on TYE-GLU-AMP plates (or TYE-GLU-KAN plates for helper phage titration) and incubated overnight at 37 °C. After recovery / titration, the phage library was diluted in PBS containing 10 % glycerol to get a final concentration between $9x10^{11}$ and $2x10^{13}$ pfu (plaque-forming units) per mL and aliquots of 0.5 mL were prepared and stored at –20 °C.

3.2.6 Methods for phage selection

Three methods were compared for phage selection. A summary of the procedures is given in figure 3.1. I performed experiments with affinity precipitation (AML having the biotinylated MUC-1). The results were compared with phage selection on immuno tubes (coated with MUC-1) and with streptavidin-coated magnetic beads (with biotinylated MUC-1), obtained

by Dorothée Dumoulin during her diploma work in our group (for both protocols see ref. [20]).

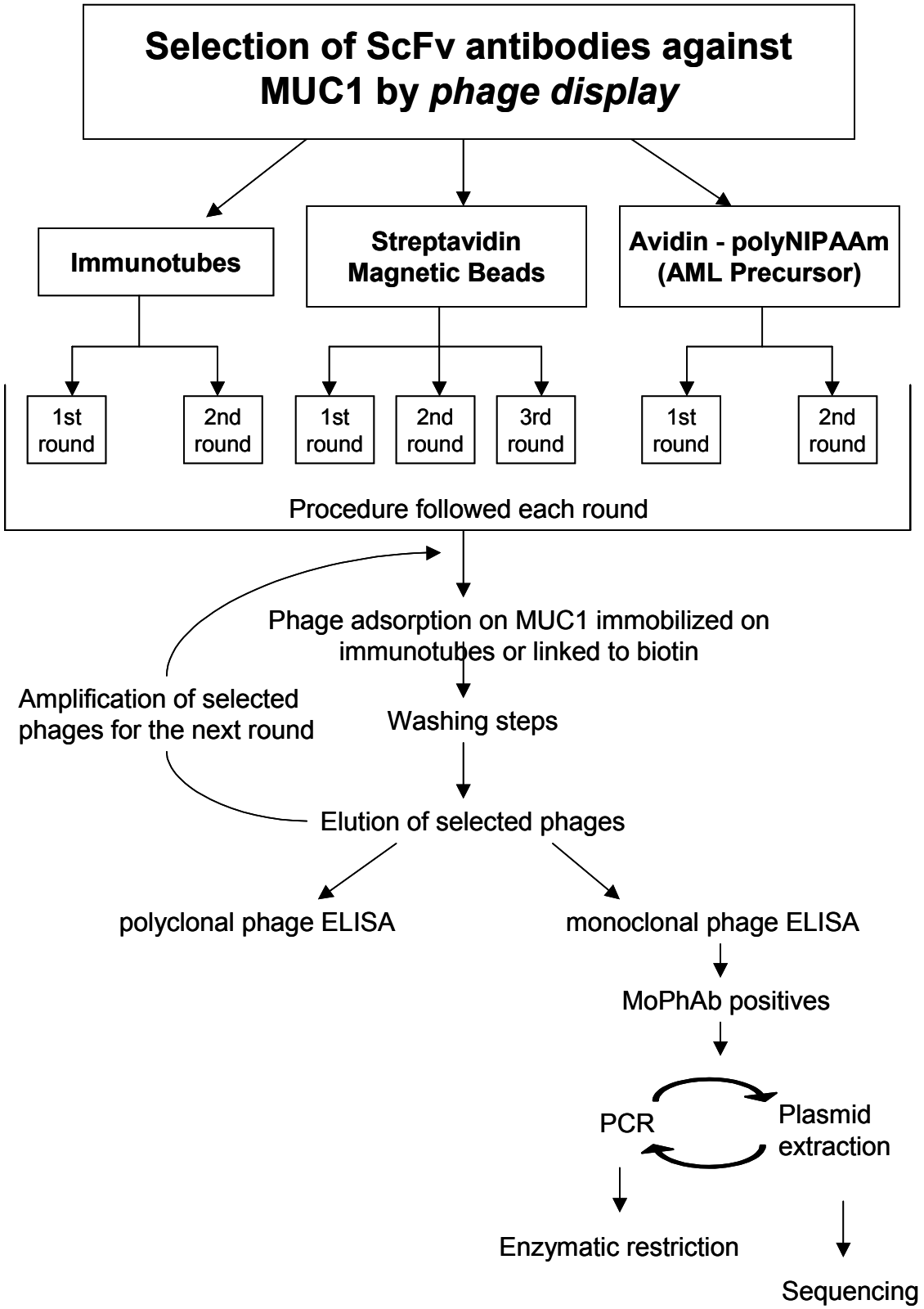


Figure 3.1. Schematic presentation of the three phage selection procedures and the means of analysis of selected phage antibodies.

3.2.6.1 Phage selection by affinity precipitation

For phage selection by affinity precipitation, stimuli-responsive bioconjugates were used essentially following the same steps as for the magnetic beads [20]. Briefly, screening of the phage library was performed using avidin-activated-polyNIPAAm (AML-precursor) to capture the biotinylated MUC1-phage complex from the solution as follows. In place of a magnetic separation stand, a thermocycling step consisting of a precipitation (40 °C, 300 rpm, 5 min) followed by centrifugation (40 °C, 10'000 *g*, 5 min) was used to recover the phages bound via the biotinylated MUC-1 to the AML. For the selection, an empty 2 mL microtube (tube 1) was filled with blocking buffer. In tube 2 300 µL and in tube 3 400 µL of AML-precursor solution (corresponding to 5 mg avidin and 27 mg polyNIPAAm / mL) in storage buffer (75 mM NaCl, 7.5 mM sodium citrate) were mixed with 4 mg respectively 5.3 mg of cellulose (Arbocel FDY 600, Size: 60 x 30 µm, J. Rettenmaier & Sohne, Rosenberg, Germany). The addition of the cellulose is optional, but accelerates redissolution of the pellet after centrifugation. The storage buffer was removed by a thermocycling step and the AML-precursor was 'washed' (twice) with 300 µL (respectively 400 µL) of PBS (washing = resuspension in cold PBS followed by a thermocycling step). Then 1 mL of cold phage solution (10^{12} phages (pfu) per ml) from the previous round (or for the first round 250 µL of each of four sub-libraries) was used to resuspend the AML-precursor-pellet in tube 2 (phages showing 'non-specific' binding to the AML-precursor will attach to it at this point and will be discarded). In addition, 16 mg of cellulose and 400 µL of a 80 mg/mL of non-activated polyNIPAAm in blocking buffer were added to tube 2 to reach a final total polyNIPAAm concentration of 3 % w/v. The AML-precursor in tube 3 was resuspended in 400 µL of cold blocking buffer, to block the non-specific binding sites. A solution of biotinylated MUC-1 peptide was prepared in tube 4 (3.5 mg/mL or 1.31 x 10^{-3} M). All four tubes were incubated overnight at room temperature on a turntable. The blocked tube 1 was emptied, and the phage-supernatant (containing the phages minus those that were bound to the AML-precursor due to non-specific interaction) recovered by a thermocycling step from tube 2 as well as 30 µL (40 nmol) of the biotinylated MUC-1 solution were transferred to tube 1 and incubated for 1 h on a turntable. The pre-blocked AML-precursor from tube 3 was recovered by a thermocycling step, resuspended in 50 µL of fresh cold blocking buffer and added to tube 1. After 15 min incubation at ambient temperature without agitation, 12 mg of cellulose and 300 µL of the cold solution of non-activated polyNIPAAm (final total polyNIPAAm concentration of 2 % w/v) were added. The phage-AML complexes were recovered by a

thermocycling step and the complexes were washed 8 times with 1.2 mL PBS-Tween and 7 times with 1.2 mL PBS. Bound phages were eluted by incubation (6 min on a turntable) with 1 mL of cold and freshly prepared 100 mM triethylamine. AML were removed by a thermocycling step and the phage-containing supernatant was immediately transferred to 0.5 mL of 1 M Tris, pH 7.4 for neutralization. Afterwards the selected phages were submitted to the 'rescue of the selected phages' protocol given below.

3.2.6.2 Rescue of the selected phages (after panning)

10 mL of an exponentially growing *E. coli* culture were infected with the 1.5 mL eluate obtained by the selection protocol given above and incubated without shaking for 30 min at 37 °C. 10 µL of this culture were retained for titration on TYE-GLU-AMP plates following the protocol described before, albeit starting directly with the serial dilution before plating. The rest of the phage solution was centrifuged (2'300 *g,* 15 min) followed by the gently resuspension of the pellet in 1 mL LB medium. This solution was plated on a Nunc Bio-Assay dish (30 cm x 30 cm) on TYE-GLU-AMP and incubated overnight at 37 °C. 10 mL of LB medium containing an additional 15 % of glycerol were added and the bacteria were gently detached and mixed with a glass spatula until a homogenous suspension was obtained. 50 mL of LB-GLU-AMP were inoculated with 80 µL of this bacterial suspension (the remaining bacteria were stored at – 80 °C) to reach an OD_{600} of 0.05 to 0.1. After the culture had reached the exponential growth phase, 10 mL were infected with helper phages in a ration of phage to bacteria of approximately 20 : 1 for 30 min at 37 °C without agitation. The infected bacteria were spun down (2'300 g, 15 min) and the pellet was gently resuspended in 100 mL LB medium followed by 90 min incubation (37 °C, 120 rpm). 100 µL of a 100 mg/mL ampicillin solution and 100 µL of a 25 mg/mL kanamycin were added and the culture incubated overnight (37°C, 120 rpm). The selected phages were recovered and titrated as described before for the phage library.

For rescue of phages for monoclonal phage ELISA, individual colonies from the plates containing phage infected bacteria spread after the desired round of selection were inoculated into 200 µL of LB-GLU-AMP medium in 96-well plates and incubated overnight at 37 °C and 300 rpm (Titramax 101 shaker). A small volume (about 2 µL) from each well was transferred to a second 96-well plate containing 200 µL of LB-GLU-AMP medium and incubated for 2 to 3 h at 37 °C and 300 rpm. Glycerol was added to the original 96-well plate to a final concentration of 15 % and the plates were stored at -20 °C. To each well of the

second plate, 25 µL of LB-GLU-AMP medium containing 10^9 pfu helper phage were added. The plate was incubated at 37 °C without agitation for 30 min, then agitated (300 rpm) for 1 h at 37 °C. The bacteria were recovered by centrifugation (1050 g, 20 min). The pellet was resuspended in 200 µL of LB-AMP-KAN medium and incubated overnight at 30 °C. The phage-containing supernatants were centrifuged (1050 g, 20 min) and used as MoPhAb in the Phage-ELISA protocol described before.

3.2.7 Preparation of Phage DNA

The phagemid DNA was extracted using the NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. 3 µL of an overnight culture of infected *E. coli* culture were harvested by centrifugation (30 s, 11'000 *g*). The pellet was resuspended in 250 µL buffer A1 from the kit by vigorous vortexing. 250 µL of buffer A2 from the kit were added and gently mixed in by inversion. The mixture was incubated 5 min followed by the addition of 300 µL buffer A3 and gentle mixing by inversion. The lysate was clarified by centrifugation (5 min, 11'000 *g)*. The supernatant was loaded on a NucleoSpin Plasmid QuickPure column and centrifuged (1 min, 11'000 *g*) for phagemid DNA binding. 450 µL AQ buffer was loaded in the column and centrifuged (4 min, 11'000 g) for washing and drying. Highly pure DNA was eluted by adding 50 µL AE buffer to the column, incubating for 1 min, and centrifuging for 1 min at 11'000 *g*.

3.2.8 PCR

The scFv gene was amplified by PCR with primers LMB3 and fdseq1 covering the 3'-portion of the VH gene, the peptide linker and the VL gene. For the amplification, 3 µL of an overnight *E. coli* culture infected with MoPhAb or 3 µL of purified phagemid DNA (obtained with the NucleoSpin Plasmid QuickPure Kit) were pipetted into a PCR reaction solution (final volume 50 µL) containing 1.25 U of *Tfl* DNA polymerase, 1x *Tfl* polymerase buffer, 1.5 mM Mg^{2+}, 1 µM of each primer, 200 µM dNTP mix and nuclease-free water (to adjust the final volume to 50 µL). The PCR was carried out with a Techne TC-512 Thermal Cycler (Techne Inc, Burlington, N.J.), and the program consisted of an incubation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 sec / 55 °C for 1 min / 72 °C for 2 min, final extension step 72 °C for 7 min. Amplicons were analysed by agarose gel electrophoresis (7 cm, 0.8 % gel in 1X TAE buffer (40 mM Tris-acetate and 1mM EDTA at pH 8.3) containing 1 µg/mL

ethidium bromide, run in a horizontal gel electrophoresis unit (Mini-Sub Cell GT system (Bio-Rad, Herkules, CA) coupled to a Bio-Rad PowerPac 300) at 80 V for 45 min. The 1 kb DNA ladder from Invitrogen was used for sizing the DNA.

For restriction analysis, the resulting PCR products were digested with *Bst*NI (New England Biolabs, Ipswich, MA) or *Ava*II (Promega). The restriction mixture (5 U of *Bst*NI and 1x NEBuffer 2 (10 mM Tris-HCl (pH 7.9 at 25°C), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol); 3 U of *Ava*II and 1x MULTI-CORE Buffer (25 mM Tris-Acetate (pH 7.5 at 37 °C), 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT) was added to the PCR mixture in a 1 : 1 ratio. The mixture was incubated (Techne TC-512 Thermal Cycler) for 2 h at 60 °C for *Bst*NI and for 2 h at 37 °C for *Ava*II. The restriction products were analysed by agarose gel electrophoresis (7 cm, 3 % gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.3) containing 1 µg/mL ethidium bromide, run in a horizontal gel electrophoresis unit (at 80 V for 45 min). Afterwards the gel was stained for 1 h in a 1 µg/mL ethidium bromide solution. The 50 bp DNA Step ladder from Promega was used for sizing the restriction products.

The nucleotide sequence was determined for me by Microsynth, Balgach, Switzerland. The provided sequences of the selected scFv antibodies were compared with the Human antibody germ line gene segments V_H DP-47, V_λ DPL-16 and V_κ DPK-22 (software: V BASE database, http://vbase.mrc-cpe.cam.ac.uk/) and the amino acid sequence of the variable V_H CDR3 region of the selected clones was determined.

3.3 Results and Discussion

3.3.1 Characterization of the stimuli-responsive bioconjugate (AML)

In order to characterize the solubility behaviour of the AML under the various conditions pertinent to the planned experiments, the CST of the polyNIPAAm-avidin conjugate was recorded for 1 % (w/v) solutions in pure Millipore water (as standard control), in the blocking buffer, in the washing buffer (PBS-Tween), in the storage buffer, and in 100 mM triethylamine (eluent). The results are shown in figure 3.2. The CST of the polyNIPAAm-avidin in water was again 32.5 °C. The values for the other solutions were slightly below the value measured in pure water, in particular triethylamine (32 °C), storage buffer (31.6 °C),

blocking buffer (31.5 °C), washing buffer (30.2 °C), but remained all above 30 °C. Given the composition and especially the salt content ('salting out'-effect [21]) of the respective solutions and taking the influence of the surfactant Tween-20 into account, these changes were to be expected. Tween is a non-ionic surfactant with a sorbitan backbone and four bulky polyoxyethylene head groups, one of which is esterified with fatty acids. Based on previous results, surfactants at concentrations above the critical micelle concentrations (CMC) raise the CST [22, 23]. In some cases such an effect is even observed below the CMC. This would explain, why the CST value in the blocking and washing buffers is higher than the one calculated for the corresponding salt concentration alone.

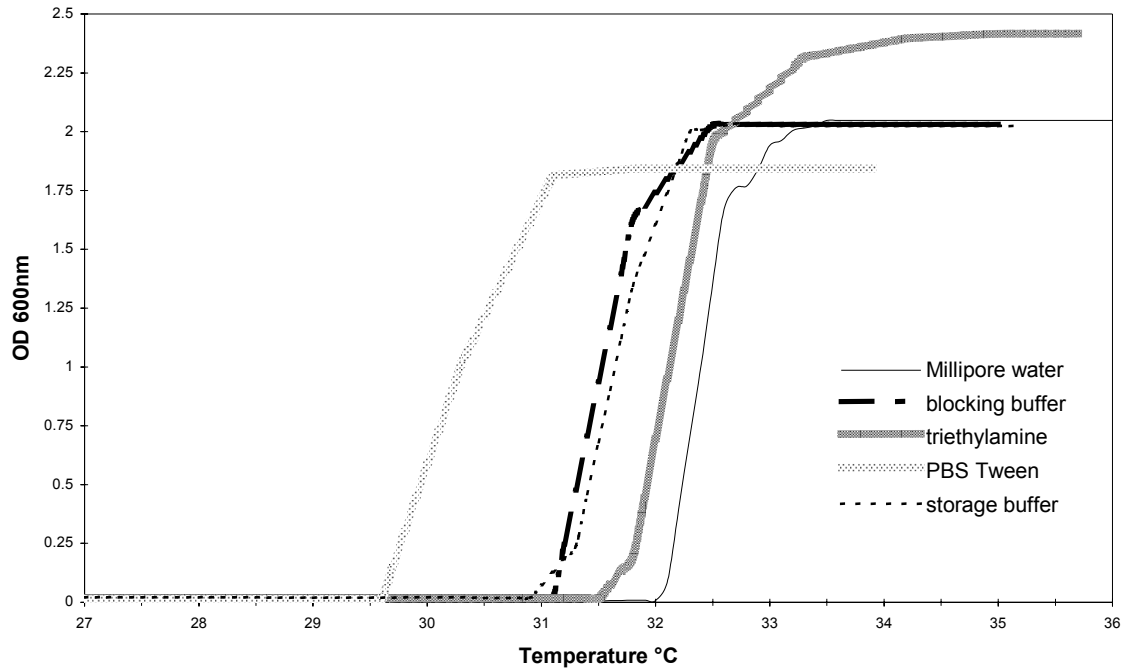


Figure 3.2. Turbidity curves recorded for 1 % (w/v) solution of polyNIPAAm-avidin in Millipore water, triethylamine, storage buffer (75 mM NaCl, 7.5 mM sodium citrate), blocking buffer (1 % (w/v) casein, 0.05 % (w/v) Tween-20 in PBS), PBS-Tween (8.2 mmol Na_2HPO_4, 1.5 mmol KH_2PO_4, 137 mmol NaCl, and 2.7 mmol KCl in 1 L water, pH 7.4, 0.1 % Tween-20).

The solubility of the AML-precursor linked to the MUC-1 peptide was not tested *per se*, but in the library screening experiments these constructs as well as the complexes containing in addition the phages showed identical precipitation / redissolution behaviour as the polyNIPAAm-avidin alone. In the subsequent affinity precipitation experiments, a temperature of 20 to 25 °C ('room temperature') was used during phage-AML affinity complex formation, while all precipitations / centrifugations were done at 40 °C, to assure quantitative precipitation. Defining the redissolution temperature required an optimization.

The lower this temperature is, the more favourable are the dissolution thermodynamics, the higher this temperature is the more favourable are the dissolution kinetics. Here a value of 4 °C was used in all steps involving AML / complex dissolution. As mentioned before, the polymer (AML) concentration, has little influence on the value of the CST in the range of interest (1 to 5 % w/v), but influences the abruptness and completeness of the precipitation [24]. Affinity precipitations in this report were all done at final polyNIPAAm concentrations of 2.0 to 3.5 % (w/v) to assure abrupt and quantitative precipitation. In order to reduce the amount of expensive AML used in the experiments to that required for capturing the target molecule, the final polyNIPAAm concentration was provided via the addition of non-activated polyNIPAAm. Since polyNIPAAm-containing pellets sometimes are difficult to redissolve after centrifugation, some cellulose fibers were added to the mixture. The cellulose does not interfere with the affinity interaction, but considerably accelerates redissolution [25].

3.3.2 Selection of phage antibodies by the investigated methods

The goal of this investigation was an evaluation of the comparative worth of affinity precipitation using stimuli-responsive bioconjugates compared to two established methods (immuno tubes, streptavidin coated paramagnetic particles (SA-PMPs)) for screening a phage library in the selection of recombinant antibodies (phage antibodies, phAb). For this purpose, scFv antibodies against MUC-1 were selected from the ETH-2 phage display library. The basic procedures for selection are outlined in figure 3.1. In the case of the AML (experiments that I realized) and the SA-PMPs (Dumoulin's experiments described in ref. [20]), a biotinylated peptide antigen was used, which was mixed beforehand into the phage suspension to allow the attachment between the phages and the peptide antigen. The complex was subsequently captured via the strong avidin-biotin interaction using either avidin-activated stimuli-responsive AML-precursor or streptavidin-activated particles. In the case of immuno tube (Dumoulin's experiments described in ref.), a non-biotinylated peptide was used to select the phages and the MUC-1 antigen was bound to the solid phase via non-covalent physisorption. After process specific incubation and washing steps using PBS-Tween and PBS as washing buffers, the bound phAb were eluted by a pH shift (100 mM triethylamine, pH 12.0). Phages selected in one round were quantified by titration and 'rescued', i.e. expanded, via infection of *E. coli*. The first round of selection was followed by one or two others. In particular, two rounds of selection were performed in case of the AML, two on the immuno tubes, and three on the SA-PMPs. The results in terms of input / output phage titres

of the various rounds are summarized in table 3.1. According to these results, input phage titres were between 1.0 x 10^9 and 8.6 x 10^{12} pfu / mL. After each round of selection, the number of eluted phages was found to decrease by several orders of magnitude, indicating that a selection of antigen-specific phages had taken place. Selection was perhaps most stringent in case of the immuno tubes, but this may be related to issues of accessibility, see discussion below.

Table 3.1. Phage titres obtained after panning of the phage library on MUC-1 using all three methods of selection.

Round of Selection		Immuno tube (pfu / mL)	Streptavidin magnetic particles (pfu / mL)	Avidin-polyNIPAAm (AML-precursor) (pfu / mL)
1	input	8.6 x 10^{12}	8.6 x 10^{12}	8.6 x 10^{12}
	output	1.6 x 10^6	1 x 10^6	2.7 x 10^7
2	input	1 x 10^{10}	4 x 10^9	7.8 x 10^{12}
	output	1 x 10^4	1.8 x 10^5	2 x 10^6
3	input	n.d.	1 x 10^9	n.d.
	output	n.d.	4 x 10^6	n.d.

n.d. : not done, pfu: plaque forming units

Phage antibodies (phAbs) from the library stock and those selected after panning using the three investigated methods (phAbs selected by immunotubes / magnetic beads were generously given by D. Dumoulin) were tested for their capacity to bind to MUC-1 peptide in a polyclonal ELISA, table 3.2. Results in terms of signal intensity (OD_{405}) were compared with those obtained using BSA (66 kDa) and Insulin (5.7 kDa) as negative controls. BSA, as a major transport protein in blood, is commonly used as negative control in binding assays, but is a very big molecule compared to the MUC-1; Insulin is closer in molecular mass and was therefore also used as mock 'antigen', see table 3.2. Insulin was not tested in case of the immuno tubes. Compared to the original library, all methods showed a pronounced increase in the binding of the selected polyclonal phAbs for the MUC-1 peptide compared to the original library already after the first round of panning. The polyclonal ELISA also demonstrated that the selected phAbs showed a more pronounced tendency for binding of the

MUC-1 peptide than for binding of either of the two negative control proteins, table 3.2. By comparison and as expected, the phAbs from the original library did not show such a preference for the MUC-1 peptide. However, compared to the original library, some increase in non-specific binding between phAbs and the negative control proteins was observed for all three methods. A possible explanation is that BSA and Insulin may show in some regions a similar amino acid configuration as the epitope in MUC-1 recognized by the phAbs. It is also possible that not enough detergent was added in the blocking buffer solution used during incubation of the phages with the coated peptide/proteins.

Table 3.2. Determination (by polyclonal phage ELISA) of the binding affinity of the phages selected using immuno tubes, beads, and stimuli-responsive bioconjugates (AML) for MUC-1, BSA, and Insulin compared to results obtained for the original library.

Coating	Original Library (a.u. 405 nm)	Immuno tube (a.u. 405 nm)		Beads (a.u. 405 nm)		AML (a.u. 405 nm)	
		Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
MUC-1	0.1138	2.3745	n.d	1.3045	1.3753	1.3483	1.2875
BSA	0.0309	0.8122	n.d	0.4575	0.5710	0.8533	0.7793
Insulin	0.0454	n.d	n.d	0.4480	0.529	0.6541	0.6288

a.u.: absorbance units, n.d. : not done

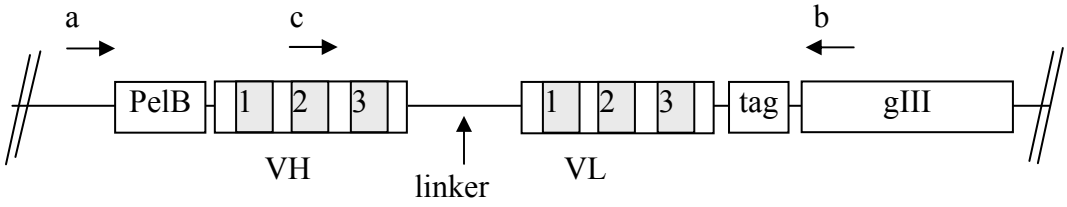
3.3.3 Diversity and sequence of the isolated phage antibodies

From the library and among the phAbs found after the corresponding round of selection, single phage clones (between 90 and 180 corresponding to one or two 96-well plates) were randomly picked out of the available monoclonal phages and tested for specific binding to MUC-1 by the monoclonal phage ELISA. In addition these clones were analysed by PCR. For this the DNA corresponding to the surface coat peptide structure was amplified by PCR using specific primers (for structures see table 3.3) and clones that in the gel electrophoresis, figure 3.3, gave amplicons of a size of 1 kb, i.e. as expected for the phAb in this case [8], were selected and marked as PCR positives. From the 96 clones picked in each the first and second round using the AML, 39 respectively 45 were ELISA positive. Altogether 6 of these were both ELISA and PCR positive. By comparison, among the 160 clones analysed from the immuno tube selection, 19 were ELISA positive. Of these 18 were PCR positive and 1 was

PCR negative. When 9 clones were randomly chosen from the 141 ELISA negative ones, 8 were PCR positive and 1 PCR negative, i.e. as to be expected for a phAb library, the Elisa negative / PCR positive clones did express phAbs, but not phAbs specific to the MUC-1 peptide antigen. In case of the screening using SA-PMPs, from 80 clones 6 were ELISA positive; 4 of which were also PCR positive and 2 PCR negative. The results are compiled in table 3.4.

Table 3.3. List of primers

Primers	Use	Position	Sequence
LMB3	PCR (forward primer)	a	5'- CAGGAAACAGCTATGAC -3'
fdseq1	PCR (reverse primer)	b	5'- GAATTTTCTGTATGAGG -3'
DP47CDR2back	sequencing	c	5'- TACTACGCAGACTCCGTGAAG-3'



In regard to the number of positive clones, it should be taken into consideration that the amount of MUC-1 peptide used for the screening was considerably higher (18 µmol, excess) in case of the immuno tubes than in case of either the beads (94 nmol) or the AML (40 nmol). This will influence the likelihood of contact with a phage and hence of selecting an interacting scFv. More disturbing is the high number of ELISA positive yet PCR negative clones. Many of these clones give amplicons of approximately 2 kb in the PCR. This may be related to the construction of the library (insertion of more than one fragment) and has been discussed before [7, 8]. Since such clones do show interaction with the antigen in the ELISA, they may putatively still be useful agents, if investigated further. The fact that such phages were more frequently picked up by the AML than by the beads and even less so by the immuno tubes, may directly be related to the accessibility of the antigen to the phages.

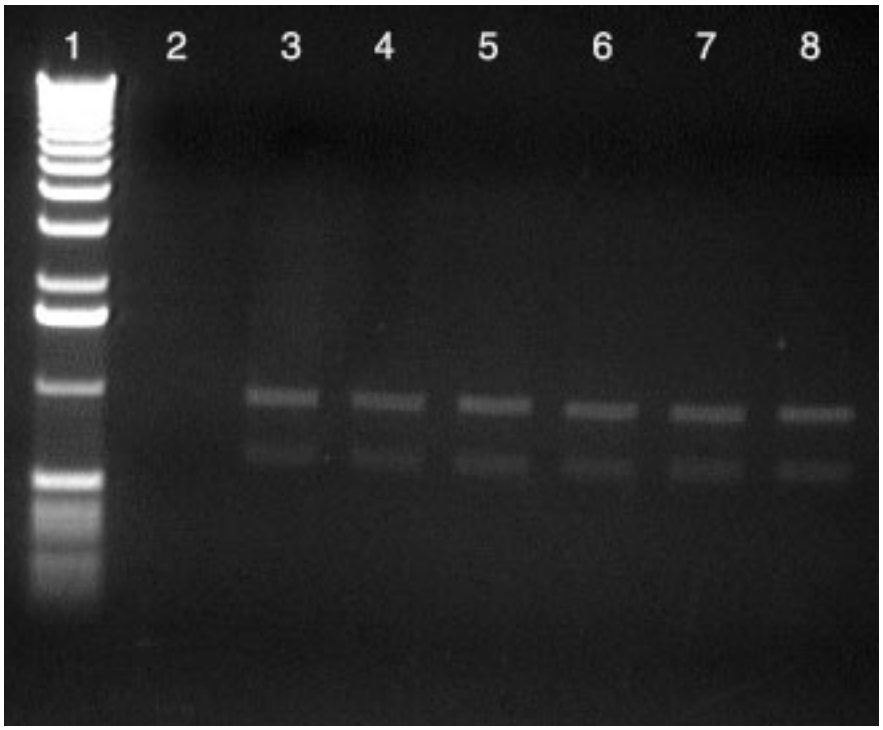


Figure 3.3. Analysis of phagemid DNA after PCR. The target DNA is known to be a 1-kb fragment from the phagemid pDN332 that contains the scFv library insert. Clones that contained such a fragment were considered PCR positive. Primers fdSeq1 and LMB3 were used for the PCR. Analysis was in a 0.8 % agarose gel run in 1x TAE buffer with 0.1 % ethidium bromide. Lanes 1: PCR marker, 1-kb DNA ladder, (Invitrogen). Lane 2: negative control (water, no plasmid DNA). Lanes 3, 4, 5, 6, 7, and 8: amplicons generated using plasmid DNA from six monoclonal phAbs (ELISA positive) selected by phage display and affinity precipitation (MoPhAb identifications are respectively G1, H1, E10, F8, C9 and G11).

In order to determine the diversity of the phAb produced by the ELISA/PCR-positive clones, these clones were further characterized by PCR restriction analysis. In case of the AML-selected phages, all 6 PCR-positive clones followed a DNA extraction procedure prior to restriction analysis. The results were compared to those obtained by Dumoulin [20] for tube / SA-beads selected clones. I should be noted that in those cases ELISA/PCR positive clones were directly analysed without doing an explicit DNA extraction step. The first restriction analysis was done with *Bst*NI. The patterns obtained for the selected clones after panning were compared with those found in the library, see below. In order to differentiate between clones having identical *Bst*NI pattern, yet a different sequence, the second restriction analysis was done using a different restriction enzyme, namely *Ava*II.

In case of the original library, eight characteristic patterns (dubbed I, II, III, IV, V, VI, VII and VIII) were found after *Bst*NI restriction analysis of the amplicons [20], figure 3.4. From the six ELISA/PCR-positive clones selected via screening with the AML, four gave a pattern corresponding to library pattern I and two gave a pattern corresponding to library pattern II in

the restriction analysis with *Bst*NI, figure 3.5. Both patterns, I and II, were also found in clones selected with the immuno tubes or the magnetic beads. In addition, some inconsistent results were obtained with these two methods. For instance in case of the immuno tubes, a pattern IV was obtained, which was ELISA negative/PCR positive, while this pattern was ELISA positive /PCR negative in case of the magnetic beads. In addition, a pattern V was found for the immuno tubes (ELISA+/PCR+), which was not obtained in any other case. The results are compiled in table 3.4.

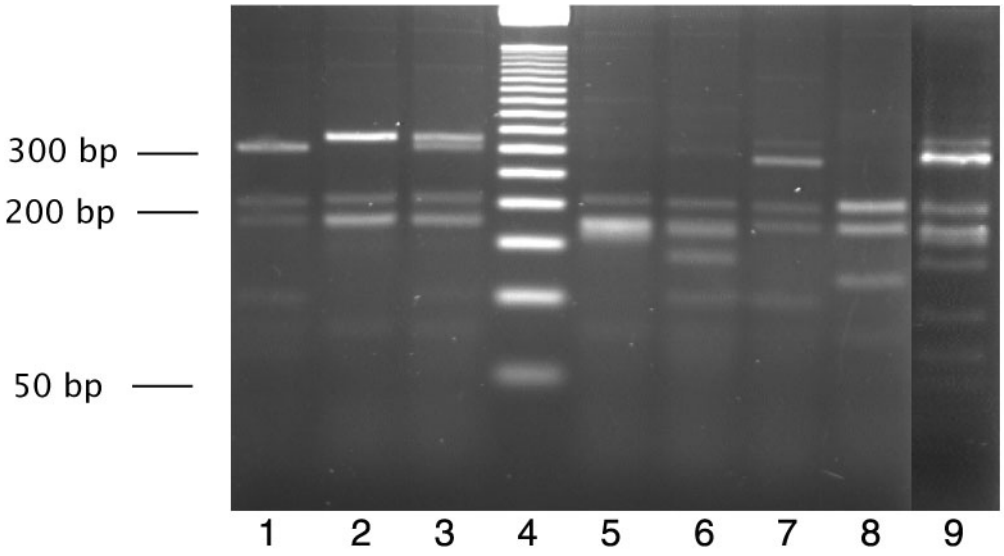


Figure 3.4. Source : [20]. Typical *Bst*NI restriction patterns found in PCR amplicons randomly produced from the phage library. For analysis, 3 % agarose gel were run in 1x TAE buffer with 0.1 % ethidium bromide. A 50 bp DNA Step Ladder (Promega) was used as size marker. Lane 1: pattern II, lane 2: pattern I, lane 3: pattern V, lane 4: size marker, lane 5: pattern III, lane 6: pattern IV, lane 7: pattern VI, lane 8: pattern VII, lane 9: pattern VIII. Note, this figure combines two gels (the first one shows lanes 1 to 8 and the second shows lane 9).

When the clones obtained with the AML that had shown a pattern I with *Bst*NI restriction, were analysed with *Ava*II after PCR amplification, two different patterns (Ia, and Id) where found, figure 3.6a, arguing that the sequence of these clones was not identical. *Ava*II restriction of ELISA/PCR positive clones with pattern I obtained with the immuno tubes gave patterns Ia, Ib, and Ic, figure 3.6b. In the case of selection via magnetic beads, only pattern Ia was found for the ELISA/PCR positive clones. Clones obtained with the AML that had a pattern II for restriction with *Bst*NI, gave two patterns (IIa and IIb) when restricted with *Ava*II after PCR amplification, figure 3.6a. In comparison, all clones obtained with immuno tubes

and magnetic beads that had pattern II when restricted with *Bst*NI, showed a pattern IIa in the case of *Ava*II. The results are compiled in table 3.4.

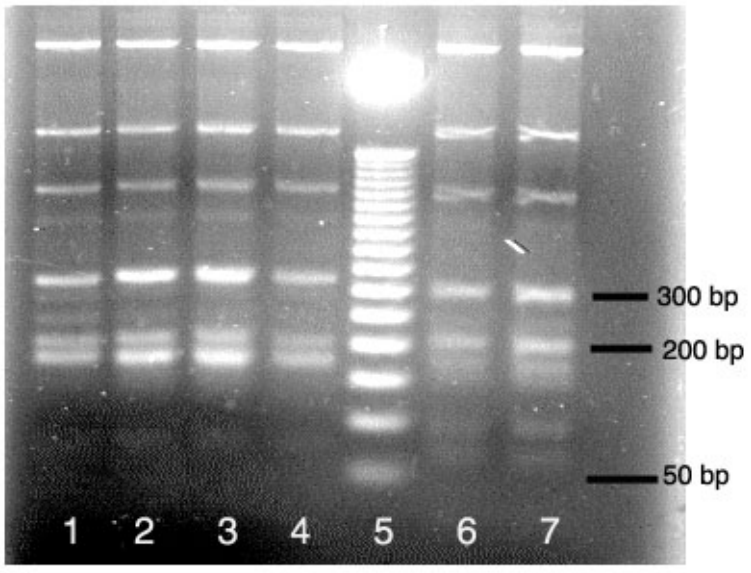
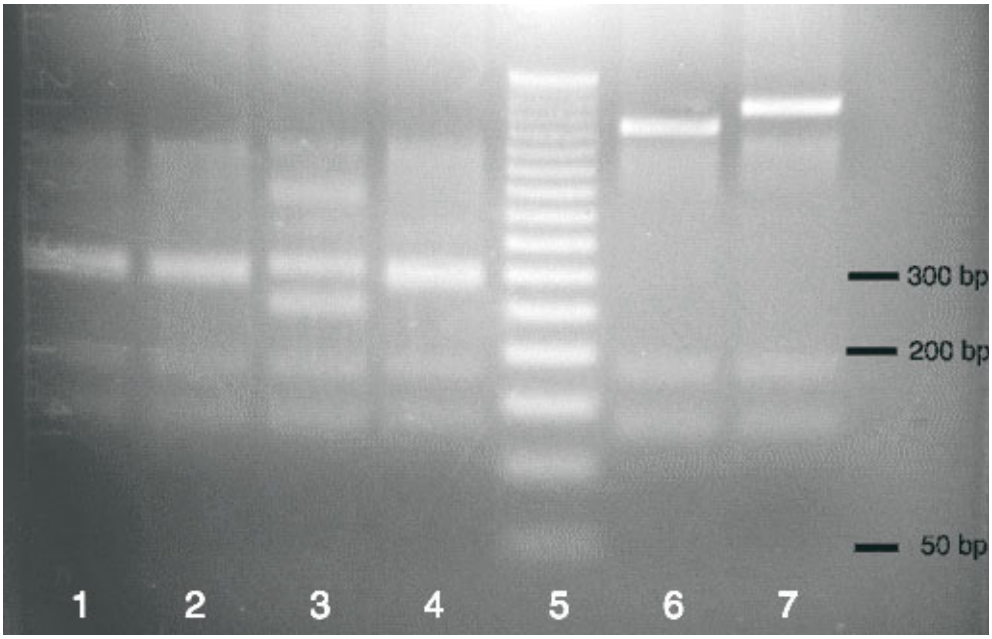


Figure 3.5. *Bst*NI restriction patterns of amplicons generated from the six monoclonal phAbs (ELISA positive, PCR positive) selected by phage display combined with affinity precipitation, showing in lanes 1 to 4 (samples G1, E10, F8 and G11 respectively) a pattern I and in lanes 6 and 7 (samples H1 and C9 respectively) a pattern II. For analysis, 3 % agarose gel were run in 1x TAE buffer with 0.1 % ethidium bromide. The 50 bp DNA Step Ladder (lane 5) from Promega was used as size marker.

In order to further elucidate the genetic differences between the selected phAbs, the V regions of the selected monoclonal phAbs were sequenced using the primer DP47CDR2back (priming in the VH germ line gene, before the V_H CDR3 region). The results are compiled in table 3.5. The sequence was analysed using the V-BASE sequence directory, a database for the human germ line V gene sequences, which comprise the ETH-2 synthetic human antibody library. The presence of the V_H chain DP-47 in all investigated clones confirmed that the library was constructed using this germ line. The expected germ lines sequences for the V_L chain, namely DPL-16 (JL2) and DPK-22 (JK1), were also found. In case of the CDR3 fragments, however, different sequences were found, sometimes even in the case of a distinct double restriction subpattern e.g. Ia.

a



b

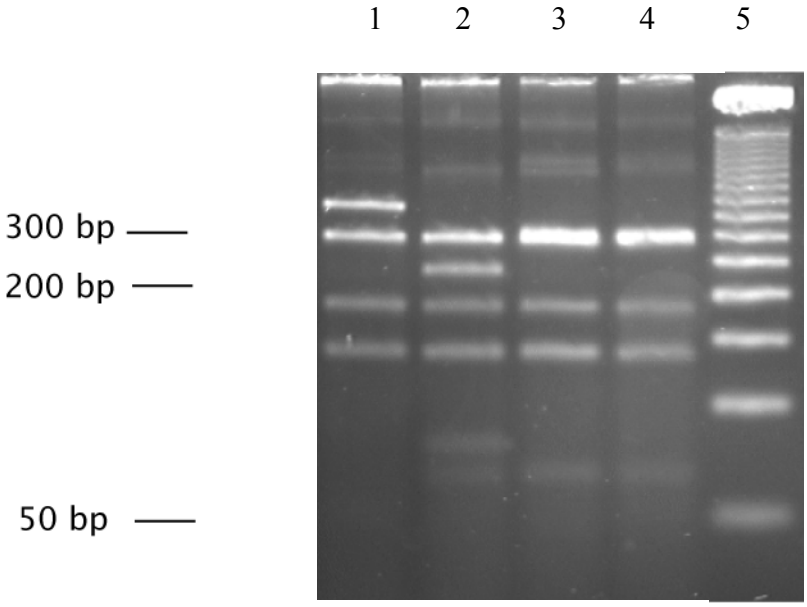


Figure 3.6. Restriction patterns found in amplicons after *Ava*II restriction analysis. For analysis, 3 % agarose gel were run in 1x TAE buffer with 0.1 % ethidium bromide. Figure 3.6a: Amplicons generated from the six monoclonal phAbs (ELISA positive, PCR positive) selected by phage display combined with affinity precipitation. Lanes 1, 2 and 4 (samples G1, E10 and G11 respectively) show a pattern Ia, lane 3: sample F8 shows pattern Id, lane 5: 50bp DNA Step Ladder (Promega), size marker, lane 6: sample H1, pattern Iia, lane 7: sample C9, pattern IIb. Figure 3.6b: reprinted from . Amplicons generated from monoclonal phAbs (ELISA positive, PCR positive) selected by phage display combined with immuno tubes showing lane 1: pattern 1b lane 2: pattern Ic, lane 3: pattern Ia, lane 4: pattern Ia, lane 5: size marker.

Table 3.4. Summary of the results of the monoclonal ELISA assay, PCR amplification and enzymatic restriction. Phage clones obtained with the three phage selection procedures were first tested against coated MUC-1, then ELISA positive clones were analysed by PCR to find clones with 1 kb amplicons, and finally PCR positive clones were analysed by BstNI or AvaII restriction analysis.

Solid phase	**Immuno tubes**	**Beads**			**AML**	
Round	1	1	2	3	1	2
Initial clones	160	80	18	104	96	96
Clones (ELISA positive)	19	6	5	15	39	45
ELISA positive if OD_{405} over:	0.2	0.1	0.1	0.1	0.03	0.1
Clones (ELISA positive and PCR amplicon 1 kb)	18	4	n.d		4	2
Clones (ELISA positive and PCR amplicon 0.7-0.8 kb)	1	0			0	0
Clones (ELISA positive and PCR amplicon >1 kb)	0	2			35	43
Pattern I # (Ia, Ib, Ic, Id)*	(5,2,1,0)	(1,0,0,0)	n.d.		(2,0,1,0)	(1,0,0,0)
Pattern II # (IIa, IIb)*	(9,0)	(2,0)			(1,0)	(0,1)
Pattern III #	0	0			0	0
Pattern IV #	0	1			0	0
Pattern V #	1	0			0	0
Pattern VI #	0	0			0	0
Pattern VII #	0	0			0	0
Pattern VIII #	0	0			0	0

n.d.: not done, #: from clones (ELISA positive and PCR positive (amplicon 1 kb)) *: Clones that showed the same restriction pattern after *Bst*NI restriction analysis, were again analysed with *Ava*II to differentiate them.

Table 3.5. Sequences obtained for the selected clones (ELISA + / PCR +)

			Germ line segments *		
	Clone	**Sequence CDR3**	**VH**	**VL**	**Pattern**
Immuno tubes Round 1	1B1	C92AKRPLLPPFDY	DP47	DPL16 (JL2)	Ia
	1D1	C92AKQTCTSPFDY	DP47	DPL16 (JL2)	Ib
	1G7	C92AKKARWKSFDY	DP47	DPL16 (JL2)	Ic
	1A5	C92AKCYSNFDY	DP47	DPL16 (JL2)	Ia
	1H11	C92AKRINKTFDY	DP47	DPK22 (JK1)	IIa
	2E7	C92AK ?	DP47	?	V (?)
Beads Round 1	5E10	Not determined	DP47	DPL16 (JL2)	Ia
	5A3	C92AKTRS?LLFDY	DP47	DPK22 (JK1)	IIa
	5H9	C92AKTRS?LLFDY	DP47	DPK22 (JK1)	IIa
	5D1	Not determined			IV
AML	**Round 1** G1	C92AKPDSKDAFDY	DP47	DPL16 (JL2)	Ia
	Round 1 H1	C92AKHstopKQstopSFDY	DP47	DPK22 (JK1)	IIa
	Round 1 E10	C92AKSKGDstop?FDY	DP47	DPL16 (JL2)	Ia
	Round 1 F8	C92AKYVTGSHFDY	DP47	DPL16 (JL2)	Ic
	Round 2 C9	C92AKFGLWPCFDY	DP47	DPK22 (JK1)	IIb
	Round 2 G11	C92AKASKLNFDY	DP47	DPL16 (JL2)	Ia

***** Reference for germ line genes see V-BASE sequence directory (http://vbase.mrc-cpe.cam.ac.uk).

When I searched for homologies between sequences in terms of size, charge, hydrogen-bridging capacity, and chemical reactivity, the amino acid serine (S, polar side chain, non-charged, with an aliphatic hydroxyl group) was found in almost all selected CDR3 sequences. In fact, the sequence serine-lysine (SK, K has a very polar side chain and is positively charged at neutral pH) was found in all clones with pattern Ia, i.e. the most consistently observed subpattern in case of pattern I for all investigated screening methods. Clones with pattern IIa encoded mostly amino acids with hydrophilic side chain, while pattern IIb was mostly hydrophobic. MUC-1 is known to present a hydrophilic immuno-dominant region (sequence PDTRP, [11]) that may be the epitope recognized by most of the selected phAbs. It also contains a strongly hydrophobic region around the valine that could be the MUC-1 epitope for phAbs of pattern IIb, i.e. the pattern only picked up by the AML. When ELISA positive yet PCR negative clones were sequenced, no sequence corresponding to a possible scFv fragment was found.

3.4 Conclusions

Stimuli-responsive bioconjugates containing relatively small polyNIPAAm segments (< 2'000 g/mol) can be used for pulling down viral particles. Under optimized conditions the process is easy to handle, requiring no dedicated equipment save for a standard laboratory centrifuge. Compared to two standard methods (selection via immuno tubes and streptavidin-coated paramagnetic particles), the use of stimuli-responsive agents results in a greater genetic variability of the selected phage antibodies, including e.g. a strongly hydrophobic epitope that was not picked up by the other two methods.

3.5 References

1. Zotter, S.; Hageman, P.C.; Lossnitzer, A.; Mooi, W.J.; Hilgers, J. Tissue and tumor distribution of human polymorphic epithelial mucin. Cancer Rev, **1988**, 11, 55-101.
2. Gendler, S.; Taylor-Papadimitriou, J.; Duhig, T.; Rothbard, J.; Burchell, J. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J. Biol. Chem., **1988**, 263, 12820-12823.
3. Berry, N.; Jones, D.B.; Smallwood, J.; Taylor, I.; Kirkham, N.; Taylor-Papadimitriou, J. The prognostic value of the monoclonal antibodies HMFG1 and HMFG2 in breast cancer. Br. J. Cancer, **1985**, 51, 179-186.
4. Winthrop, M.D.; DeNardo, S.J.; DeNardo, G.L. Development of a hyperimmune anti-Muc-1 single chain antibody fragments phage display library for targeting breast cancer. Clin. Cancer Res., **1999**, 5, 3088-3094.
5. Barratt-Boyes, S.M. Making the most of mucin: a novel target for tumor immunotherapy. Cancer Immunol Immunother, **1996**, 43, 142-151.
6. Smith, G.P. Filamentous fusion phage - Novel expression vectors that display cloned antigens on the virion surface. Science, **1985**, 228, 1315-1317.
7. de Bruin, R.; Spelt, K.; Mol, J.; Koes, R.; Quattrocchio, F. Selection of high-affinity phage antibodies from phage display libraries. Nature Biotechnology, **1999**, 17, 397-399.
8. de Greeff, A.; van Alphen, L.; Smith, H.E. Selection of recombinant antibodies specific for pathogenic *Streptococcus suis* by substractive phage display. Infection and Immunity, **2000**, 68, 3949-3955.
9. Kontermann, R.E., Single-chain Fv fragments from phage display libraries, in Vector Targeting for Therapeutic Gene Delivery, D.T. Curiel and J.T. Douglas, Editors. **2002**, John Wiley & Sons, Inc.
10. Marks, J.D.; Hoogenboom, H.R.; Bonnert, T.P.; McCafferty, J.; Griffiths, A.D.; Winter, G. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol., **1991**, 222, 581-597.
11. Wong, C.; Waibel, R.; Sheets, M.; Mach, J.-P.; Finnern, R. Human scFv antibody fragments specific for the epithelial tumor marker MUC-1, selected by phage display on living cells. Cancer Immunol Immunother, **2001**, 50, 93-101.
12. Pini, A.; Viti, F.; Santucci, A.; Carnemolla, B.; Zardi, L.; Neri, P.; Neri, D. Design and use of a phage-display library: human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. J. Biol. Chem., **1998**, 273, 21769-21776.
13. Winter, G.; Griffiths, A.D.; Hawkins, R.E.; Hoogenboom, H.R. Making antibodies by phage display technology. Annu. Rev. Immunol., **1994**, 12, 433-455.
14. de Haard, H.J.; van Neer, N.; Reurs, A.; Hufton, S.E.; Roovers, R.C.; Henderikx, P.; de Bruïne, A.P.; Arends, J.-W.; Hoogenboom, H.R. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem., **1999**, 274, 18218-18230.
15. Winthrop, M.D.; DeNardo, S.J.; Albrecht, H.; Mirick, G.R.; Kroger, L.A.; Lamborn, K.R.; Venclovas, C.; Colvin, M.E.; Burke, P.A.; DeNardo, G.L. Selection and characterization of anti-MUC-1 scFvs intended for targeted therapy. Clin. Cancer Res., **2003**, 9, 3845-3853.
16. Stocker, G.; Vandevyver, C.; Hilbrig, F.; Freitag, R. Purification of RT-PCR competent poly(A) mRNA from crude cell lysate by affinity precipitation. Biotechnol. Prog., **2006**, 22, 1621-1629.

17. Garret-Flaudy, F. Thermoresponsive oligomers - solubility and applications for affinity precipitation, **2000**, PhD Thesis EPFL, Lausanne
18. *ETH-2 library*. Available from: http://www.pharma.ethz.ch/institute_groups/biomacromolecules/protocols/ETH2.doc.
19. Hoogenboom, H.R.; Griffiths, A.D.; Johnson, K.S.; Chiswell, D.J.; Hudson, P.; Winter, G. Multisubunit proteins on the surface of filamentous phage - methodologies for displaying antibody (fab) heavy and light chains. Nucleic Acid Res., **1991**, 19, 4133-4137.
20. Stocker, G.; Dumoulin, D.; Vandevyver, C.; Hilbrig, F.; Freitag, R. Screening of a synthetic human antibody phage display library against the MUC-1 surface antigen using smart bioconjugates. Biotechnol Prog., **submitted**,
21. Freitag, R.; Garret-Flaudy, F. Salt effects on the thermoprecipitation of poly-(N-isopropylacrylamide) oligomers from aqueous solution. Langmuir, **2002**, 18, 3434-3440.
22. Garret-Flaudy, F.; Freitag, R. Unusual thermoprecipitation behavior of poly-(N,N-diethylacrylamide) from aqueous solution in the presence of anionic surfactants. Langmuir, **2001**, 17, 4711-4716.
23. Garret-Flaudy, F.; Freitag, R. Influence of small amphiphilic on the Lower Critical Solution Temperature (LSCT) of highly homogeneous N-alkylacrylamide oligomers. J. Polym. Sci. A, **2000**, 38, 4218-4229.
24. Hilbrig, F.; Freitag, R. Protein purification for affinity precipitation. J. Chromatogr., B, **2003**, 790, 79-90.
25. Freitag, R.; Schumacher, I.; Hilbrig, F. Affinity precipitation an option for early capture in bioprocessing Biotechnol. J., **2007**, 2, 685-690.

4. Human Haptoglobin-PolyNIPAAm as AML for Affinity Precipitation of Human Hemoglobin

4.1 Introduction

Hemoglobin (Hb) is the major component of red blood cells, and is responsible for their red color (For reviews or general information about Hb, see references [1-4]). It is the most important respiratory protein of vertebrates by virtue of its ability to transport oxygen from the lungs to body tissues, and to facilitate the return transport of carbon dioxide. Hb is a tetramer composed of 2 pairs of polypeptide chains called globins, and 4 heme groups (containing one iron / heme group). Each polypeptide chain carries one heme. Hb A, the major hemoglobin (98 %) in the blood of adults, is composed of two α and two β chains. Mammalian hemoglobins have molecular weights of 64.5 kDa [1]. Ferrihemoglobin, also called methemoglobin (MHb), contains iron in the +3 oxidation state (Fe^{3+}); Ferrohemoglobin, also called reduced hemoglobin, contain iron in the +2 oxidation state (Fe^{2+}). Hemoglobin must be in the reduced form to bind oxygen or other small molecules, i.e., carbon monoxide or cyanide [2].

Haptoglobin (Hp) is a glycoprotein (also called α_2-globulin) synthesized primarily in the liver (hepatocytes) and found in normal plasma of many animal species (For reviews about Hp, see [5-7]). The monomeric form of this acute phase plasma protein is composed of four polypeptide chains: two α chains and two β chains. Hp is polymorphic due to the existence of two allelic forms of the α chains (α-1 and α-2). Human Hp occurs naturally as 3 major phenotypes: Type 1-1 (2 α-1 / 2 β, 100 kDa), Type 1-2 (α-1 α-2 / 2 β, 100 up to 1000 kDa) and Type 2-2 (2 α-1 / 2 β, 100 up to 1000 kDa) [8, 9]. Hp is a serum protein which binds Hb released by intravascular hemolysis (during inflammation or trauma) [10]. Human Hp is known to bind human Hb, with different ligands (oxy-, CO, met-, and cyanomethemoglobins) and several human Hb variants [11-15]. In contrast to ligand-bound hemoglobin, ligand-free hemoglobin (i.e., d-Hb, deoxyhemoglobin) does not react at all, or at least not at any measurable speed [16-18]. The heme group is not required for complex formation since Hp will bind globin (i.e. the protein moiety of Hb) [11, 12]. The Hp-Hb complex is removed from the bloodstream by the liver and then metabolized by the body, i.e., this binding prevents iron loss in the urine and also protects the kidneys from damage by Hb, while making the Hb

accessible to degradative enzymes [10, 19, 20]. The molecular interactions between Hb and Hp are among the strongest of all known non-covalent protein-protein interaction [21]. In this context, α chains of Hb have a much higher affinity for Hp than β chains, and it is possible, at sufficiently high chain concentration, to attain saturation of the Hp with four Hb α chains per Hp molecule [18, 22]. Laurell and Grönvall suggest that the binding of Hb to Hp occurs through the α,β dimer of Hb [11]. Some publications have found that the complex which is formed between Hp and Hb, involves the β chains of the Hp as binding site for the Hb where they can be assumed to exist as two similar independent binding sites, one β chains for each αβ hemoglobin dimer [13, 23]. Although the mechanism of the interaction of human Hp and Hb A has partially been elucidated, the nature of the binding sites on the Hp and of the bond or bonds linking Hp and Hb is still unknown [10]. In any case, it is well established that Hp binds Hb specifically, with high affinity (association constant $K > 10^{15}$ [24]) in a final molar ratio of 1:1 between Hp tetramer and Hb tetramer [12, 18, 22]. The reaction is essentially irreversible in non-denaturating buffers over a wide pH range (4.4 to 10) [12, 25]. Hp immobilized on agarose matrix preserves essentially the same interacting characteristics with Hb as does Hp in solution [22].

In the present study I have developed an affinity precipitation protocol using Hp bound covalently to polyNIPAAm as AML to purify Hb from a partially-purified solution (Hb liberated from red blood cells and precipitated by ammonium sulphate). A comparison was done with a chromatographic method using Hp bound covalently to sepharose. The influence of the coupling ratio of polyNIPAAm to Hp (AML construction), and different binding, elution and precipitation (influence of ammonium sulphate and polyNIPAAm concentration) conditions for the affinity precipitation were investigated.

4.2 Materials and Methods

4.2.1 Materials

Haptoglobin, ammonium sulphate, sodium dithionite, formic acid, buffers salts and organic solvents were from Sigma-Aldrich. Fresh human blood was obtained from the Unité de medicine transfusionnelle (UMT) at the CHUV, Lausanne. Millipore water was used to prepare the aqueous solutions unless indicated otherwise.

4.2.2 Analytical methods

All Hb and Hp concentrations are expressed as tetramer equivalents (not as heme equivalents). All absorbance measurements were done in a Lambda 20 spectrophotometer (Perkin Elmer, Norwalk, CT). The Hp content of a given sample was estimated spectrophotometrically by UV absorption at 280 nm. As the stock sample from Sigma had an unknown phenotype-composition, the molar extinction coefficient of Hp was estimated by measuring the absorbance at 280 nm of four different dilutions of a stock solution of 1 mg Hp in 300 µL water and assuming that most of the Hp present was of the phenotype Hp1-1 (MM = 100 kDa).

The Hb, more exactly oxyhemoglobin, content of a given sample was estimated spectrophotometrically by UV absorption at 415 nm with a molar extinction coefficient of 500 mM^{-1} cm^{-1} per oxyhemoglobin tetramer (125 mM^{-1} cm^{-1} per heme [26]) and also by the Drabkin's method that determines Hb concentrations in the blood by cyanomethemoglobin (MHbCN) absorbance [27]. Briefly, 20 µL of a Hb sample were added to 5 mL of Drabkin's solution (12 mM sodium bicarbonate, 0.6 mM $K_3Fe(CN)_6$ (potassium ferricyanide), 0.8 mM KCN (potassium cyanide)), mixed by swirling and incubated for 10 min before the cyanomethemoglobin absorbance was determined at 540 nm (maximum absorbance peak for cyanomethemoglobin). A molar extinction coefficient of 44 mM^{-1} cm^{-1} per cyanomethemoglobin tetramer (11 mM^{-1} cm^{-1} per heme [26, 27]) was used while the Drabkin's solution alone served as blank. A variation of the Drabkin test, called special Drabkin test, was also performed following the molar ratio indicated by Balla [15]. Briefly, methemoglobin was prepared by incubation of Hb with a 1.5-fold molar excess of $K_3Fe(CN)_6$ over heme and finally cyanomethemoglobin was obtained by the addition of a 2-fold excess KCN over heme, and the absorbance at 540 nm of MHbCN was measured.

The protein content in the elution sample was quantified using the NanoOrange Protein Quantitation Kit from Molecular Probes (Leiden, The Netherlands), according to the manufacturer's instructions. Samples were diluted in NanoOrange diluent and prepared in microtubes. The fluorescence was quantified in a microplate fluorometer (Cytofluor 4000, PerSeptive Biosystems, Foster City, CA) using Ex: 485 nm and Em: 590 nm. Standard curves were generated using bovine serum albumin (BSA, Sigma-Aldrich). The presence of Hb in the elution samples was verified by electrospray ionization mass spectrometry with time-of-

flight detection by a LCT mass spectrometer from Micromass (Manchester, UK). A preliminary gel filtration of these samples before MS analysis was done on PD-10 columns (Amersham) according to the manufacturer's instructions (see protocol "size exclusion chromatography by gravity flow" below), against deionized water. Each Hb-gel filtrated sample (total recovered volume 3.5 mL) was collected in 0.5 mL aliquots and the absorbance at 415 nm was measured to select the aliquots containing the highest amount of Hb for the MS analysis.

4.2.3 Size exclusion chromatography by gravity flow

PD-10 columns (disposable desalting column / Sephadex G-25 Medium, Amersham) were used for filtration of samples according to the manufacturer's instructions [28]. Briefly, the column was equilibrated with 25 mL appropriate elution buffer, i.e., the buffer in which the sample will be needed afterwards, and the flow-through was discarded. 2.5 mL of the sample were loaded into the column and the flow-through was discarded. 3.5 mL elution buffer were loaded into the column and the flow-through was collected for further analysis. To recover the low molecular weight substances from the column, 6 mL of elution buffer were added for washing and the flow-through was collected for further analysis. The column was additionally washed with 19 mL elution buffer and the flow-through was discarded.

4.2.4 Extraction of human hemoglobin from blood

Hb was prepared as described by Liau [29]. Briefly, fresh human blood (~520 mL) was centrifuged at 3'000 *g*, 4 °C for 25 min, afterwards the plasma was removed by aspiration. The remaining red blood cells (~260 mL) were washed five times with three volumes of phosphate-buffered saline containing 0.12 M NaCl and 12.5 mM phosphate, pH 7.2, named PBS-1 and then the cells were lysed with two volumes of deionized water at 4 °C. Cell debris was removed by centrifugation at 3'500 *g*, 4 °C for 30 min. The supernatant was then fractionated with ammonium sulphate at 20 % and 80 % saturation at 0 °C as indicated by Tsuruga [30]. Briefly, precipitates of the first precipitation step (at 20 %) were discarded by centrifugation at 3'000 *g*, 4 °C for 30 min, and the supernatant obtained was submitted to a second precipitation step (at 80 %) where the precipitated Hb was recovered by centrifugation at 10'000 *g*, 4 °C for 30 min. The Hb was redissolved in 520 mL deionized water (same volume as blood sample received from CHUV). From this solution, 37 mL were dialysed

(SnakeSkin Pleated Dialysis Tubing, 3'500 MWCO, Pierce, Rockford, IL) against deionized water or aliquots of 2 mL were gel filtrated on a PD-10 column (Amersham, see protocol described before) using deionized water or 0.1 M phosphate buffer pH 6.0 or PBS (8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as elution buffer. The other portion was kept at –20 °C until further use.

4.2.5 Reduction of hemoglobin

For the reduction of hemoglobin PD-10 columns (Amersham) were used with a slight modification of the manufacturer's instructions [28] according to the procedure described by Dixon [31]. Briefly, the column was equilibrated at room temperature with 25 mL phosphate buffer (0.1 M phosphate, pH 6.0) and the flow-through was discarded. 0.2 mL (20 mg, 0.1148 mmol) sodium dithionite were loaded on the column followed by 0.2 mL phosphate buffer and finally 2.1 mL of a solution containing 35 mg Hb/mL (0.54 mM Hb) in water for a final loaded volume of 2.5 mL and a molar ratio sodium dithionite : Hb of 100:1. The flow-through was discarded. 3.5 mL phosphate buffer were loaded into the column and the flow-through was collected in 0.5 mL aliquots. The column was washed with 25 mL phosphate buffer. Collected samples were oxygenated (to obtain HbO_2) by bubbling with O_2 for 5 min and the efficiency of the Hb reduction was checked by absorption scanning between 400 and 600 nm. All Hb stocks used for affinity precipitations were reduced and oxygenated before binding tests.

4.2.6 Synthesis of the AML

The stimuli-responsive polymer used to construct the AML was as described previously in chapter 2 (see also ref. [32, 33]) and obtained from polyTag Technology, Männedorf, Switzerland. Hp was linked to the AML-precursor by carbodiimid coupling as described by Chen and Hoffmann [34]. For this the NHS-activated polyNIPAAm was obtained following the same protocol as indicated in chapter 2 (see also ref [32]). Five AML (AML I, AML II, AML III, AML IV, AML V) were constructed with different molar ratio between polyNIPAAm and Hp (table 4.1). For Hp coupling in AML I, 4.5 mg of activated polyNIPAAm (220 mol equiv) were dissolved in 20 µL of dry DMF and added incrementally at 4 °C to a gently stirred solution of 1 mg Hp in 275 µL of a 0.2 M borate buffer (pH 8.7). In the case of AML II to V a 0.01 mg/mL activated polyNIPAAm solution in DMF was prepared

and following the same coupling procedure as for AML I, 50, 25, 5 and 2 µL respectively were added to a solution of 0.2 mg Hp in 100 µL of a 0.2 M borate buffer (pH 8.7). The solutions were gently stirred overnight at 4 °C. The next morning, 20 mg (respectively 12, 10, 9, and 8 mg for AML II to V) of non-activated polyNIPAAm were mixed into 290 µL (respectively 145, 125, 110, and 105 µL for AML II to V) of water and incubated with the Hp solution (total polyNIPAAm concentration: 3.9-4.2 % w/v). The AML was recovered by a thermocycling step (precipitation with ¼ vol $(NH_4)_2SO_4$-saturated solution at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). At this step, the amounts of saturated aqueous ammonium sulphate solution added were 146, 74, 63, 54, and 52 µL respectively for AML I to V, and the final polyNIPAAm concentration was 3.4 % w/v. The non-coupled activated polyNIPAAm was blocked when the AML was redissolved in fresh 0.2 M borate buffer, pH 8.7 at 4 °C with ethanolamine (final concentration of 0.5 M ethanolamine) followed by a thermocycling step. The AML was then redissolved in fresh 0.15 M NaOH solution at 4 °C (hydrolisation of NHS) followed by a thermocycling step. The AML was purified by repeated thermocycling in fresh 0.1 M phosphate buffer, pH 6.0 at 4 °C. The Hp-content in the AML was determined spectrophotometrically at 280 nm.

Table 4.1. Summary of the AML synthesis steps.

Coupling step	AML I	AML II	AML III	AML IV	AML V
activated polyNIPAAm (mg)	4.5	$5x10^{-4}$	$2.5x10^{-4}$	$5x10^{-5}$	$2x10^{-5}$
in DMF (μL)	20	50	25	5	2
Hp (mg)	1	0.2	0.2	0.2	0.2
in 0.2 M borate buffer, pH 8.7 (μL)	275	100	100	100	100
activated polyNIPAAm / Hp (mol/mol)	220 : 1	100 : 1	50 : 1	10 : 1	4 : 1
non-activated polyNIPAAm added (mg)	20	12	10	9	8
water added (μL)	290	145	125	110	105
% w/v total polyNIPAAm	4.2	4.1	4.0	4.2	3.9

Protocol: 1) coupling, 2) non-activated polyNIPAAm addition, 3) thermocycling, 4) ethanolamine addition, 5) thermocycling, 6) NaOH addition, 7) thermocycling, 8) washing, 9) thermocycling, 10) recovery of the AML in the desired solution.

4.2.7 Preparation of haptoglobin-activated sepharose

Hp was coupled to NHS-Activated Sepharose 4 Fast Flow (Amersham) by a slight modification of the manufacturer's instructions [35]. Briefly, a column (diameter: 12.5 mm, height: 17 mm) was packed with 2 mL (32-46 μmol NHS) NHS-activated sepharose (particle size range: 45-165 μm), column volume 2 mL) and washed with 12 mL (6 column volumes) of cold 1 mM HCl (the storage buffer, 100 % isopropanol, was here removed). 1 mg (1×10^{-5} mmol) of Hp was dissolved in 1 mL of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8) and the absorbance at 280 nm was determined. The Hp solution was loaded into the NHS-activated sepharose column (volume ratio of coupling solution to medium was 0.5 : 1) and incubated overnight at 4 °C. Non-coupled Hp was recovered by washing the sepharose with 2 mL (1 column volume) coupling buffer, then this solution was passed through a PD-10 column as described before, prior to measuring the absorbance at 280 nm for calculating the coupling yield. This step is necessary to take out the NHS present in the solution, which

absorbs strongly at 260 nm and therefore disturbs the measurement at 280 nm. Non-reacted NHS groups on the Sepharose were blocked with 12 mL (6 column volumes) of blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) for 2 h at room temperature. Two different buffers were alternately added (high pH / low pH) to the column for washing the medium after coupling, with 12 mL (6 column volumes) of a high pH washing buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3), and with 12 mL (6 column volumes) of a low pH washing buffer (0.1 M acetate buffer, 0.5 M NaCl, pH 4). This washing step cycle (alternating high / low pH buffers) was repeated two additional times. The column was kept in PBS for further use.

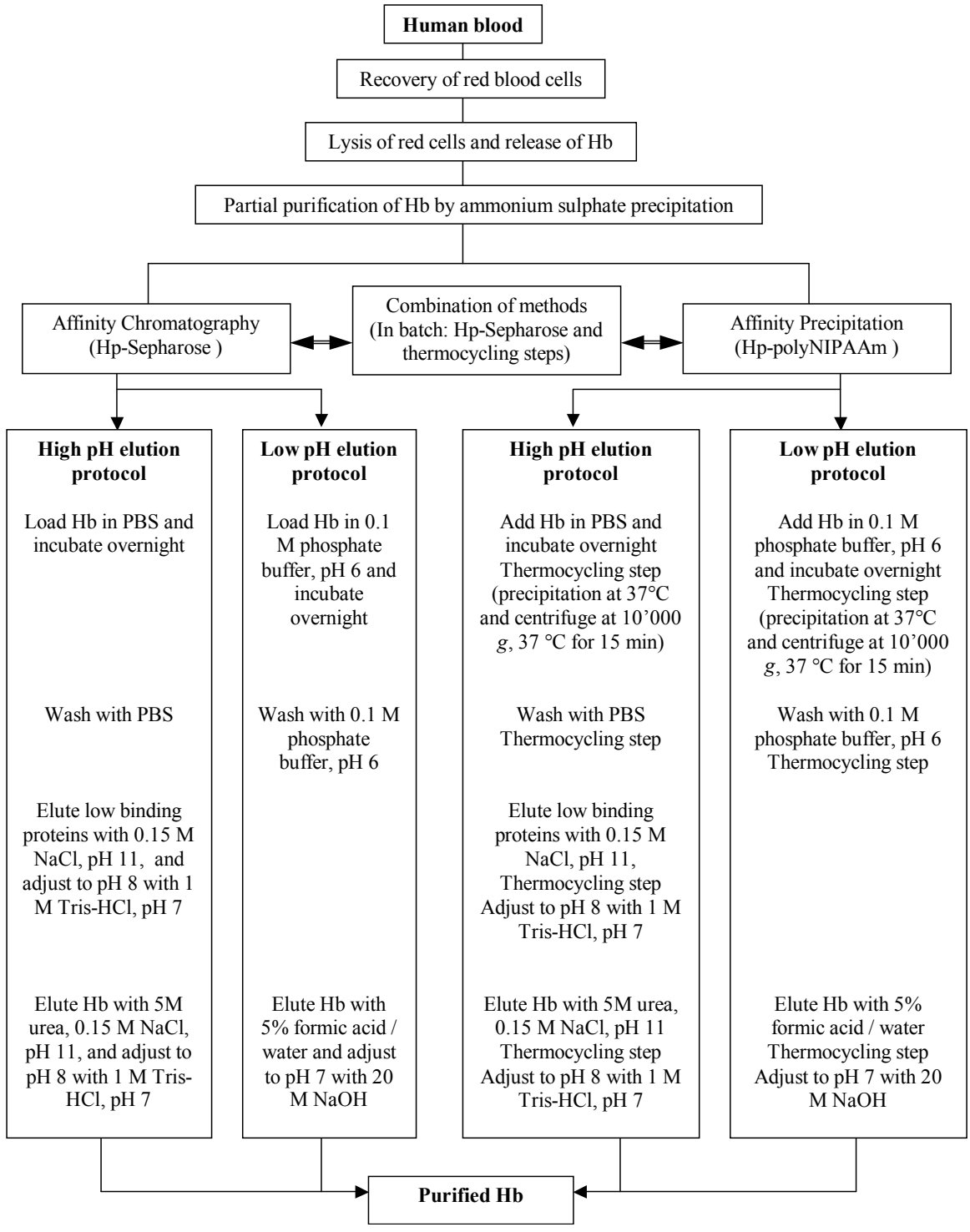


Figure 4.1. Scheme of the hemoglobin (Hb) purification procedures. Affinity precipitation was compared to affinity chromatography and to batch adsorption (using Hp-Sepharose, yet mimicking affinity precipitation conditions). Two elution protocols (high and low pH) were used for each method.

4.2.8 Isolation of hemoglobin by haptoglobin-affinity chromatography

The procedure used for affinity chromatography with high pH elution is summarized in figure 4.1. Binding, washing and elution conditions were realized as suggested by Liau [29]. The Hp column was equilibrated with 6 mL (3 column volumes) of PBS. 2 mL (1 column volume) of reduced and oxygenated Hb (1.1×10^{-3} mmol Hb, 115 fold excess of Hb related to Hp coupled in the column) in PBS were loaded into the column and incubated overnight at 4 °C. The column was washed with 24 mL (12 column volumes) of PBS. The bound materials were first eluted (elution-1) with 6 mL (3 column volumes) of 0.15 M NaCl, pH 11 (adjusted with ammonium hydroxide), where 2 mL aliquots labeled 2, 3 and 4 were recovered. In a second step, elution-2, 6 mL (3 column volumes) of 5 M urea in 0.15 M NaCl, pH 11 (freshly prepared and filtered) were added, and 2 mL aliquots labeled 5, 6 and 7 were recovered. The pH of the elution-aliquots (2 to 7) was immediately adjusted to 8.0 by adding 0.26 mL 1 M Tris-HCl, pH 7.0. The column was washed with 16 mL PBS and stored at 4 ˚C in PBS containing 0.05 % NaN_3 as preservative. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm.

The procedure used for affinity chromatography with low pH elution (see figure 4.1) was developed from results published by Shim [36], Beisembaeva [37] and Hensley [17] for binding conditions and by Tsapis [38] and Asakawa [39] for elution conditions. The Hp column was equilibrated with 6 mL (3 column volumes) binding buffer (0.1 M phosphate buffer, pH 6.0). 2 mL (1 column volume) of reduced and oxygenated Hb (1.1 x 10-3 mmol Hb, 115 fold excess of Hb related to Hp coupled in the column) in binding buffer were loaded into the column and incubated overnight at 4 °C. The column was washed with 24 mL (12 column volumes) binding buffer. The bound material was eluted with 8 mL (four column volumes) of 5 % formic acid pH 2, and 2 mL aliquots were collected in tubes labeled 2, 3 and 4 respectively. The pH of the elution-aliquots (2 to 4) was immediately adjusted to pH 7 by adding 0.125 mL of 20 M NaOH. The column was washed with 16 mL binding buffer and stored at 4 ˚C in binding buffer containing 0.05 % NaN_3 as preservative. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm.

4.2.9 Isolation of hemoglobin by haptoglobin-sepharose batch adsorption mimicking affinity precipitation conditions

Initially these experiments followed the same steps as indicated before for the affinity chromatography. However, instead of using the packed column, Hp-sepharose (2 mL, 9.6 x 10^{-6} mmol Hp) was taken out of the column and split into four microtubes (0.5 mL bed volume / tube) and subsequently subjected to batch adsorption mimicking the affinity precipitation conditions following the high pH elution (tubes 1 and 2) or the low pH elution (tubes 3 and 4) protocols (see summary of the procedures in figure 4.1). This approach allowed the investigation of the effect of the thermocycling steps (precipitation/centrifugation conditions described on the Synthesis of the AML section, but without adding ammonium sulphate) later to be used in affinity precipitation using a familiar adsorbent. 1.5 mL (0.025 mM) Hb in binding buffer (PBS or 0.1 M phosphate buffer pH 6.0), were added per microtube and the mixture was incubated at 4 °C overnight under gently mixing on a turntable. A mock thermocycling step was done ("mock precipitation" at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). For washing, the Hb-charged Hp-sepharose was gently mixed for 10 min on a turn table at room temperature in 1.5 mL fresh binding buffer (PBS or 0.1 M phosphate buffer pH 6.0) per microtube following with a mock thermocycling step. The supernatant was discarded and this washing-thermoprecipitation was repeated four times. For tubes 1 and 2, an elution step of low-affinity bound proteins was done with 1.5 mL fresh 0.15 M NaCl, pH 11 per microtube following with a mock thermocycling step. The supernatant was recovered and immediately adjusted to pH 8 with 1 M Tris-HCl, pH 7. For elution of the Hb, fresh elution buffer was used (1.5 mL of 5 M urea in 0.15 M NaCl, pH 11 or 1 mL of 5 % (v/v) formic acid in water (pH 2) per microtube) following with a mock thermocycling step, recovery of the supernatant and immediate adjustment of the pH to pH 8 with 1 M Tris-HCl or to pH 7 with 20 M NaOH. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm.

4.2.10 Isolation of hemoglobin by haptoglobin-polyNIPAAm affinity precipitation

For affinity precipitation of Hb again both high pH and low pH elution were used. In case of the high pH elution, initially the experiments followed the same steps as indicated above for the Hp-sepharose batch adsorption, see summary of the procedure in figure 4.1. However,

instead of the Hp-sepharose, the Hp-polyNIPAAm (AML I to V) was used to capture the Hb. Briefly, 100 µL of AML I-solution (1.82 µg Hp/µL, 50 µg polyNIPAAm /µL, in PBS), respectively 250 µL (0.33 µg/µL, 24 µg /µL), 100 µL (0.3 µg/µL, 40 µg/µL), 50 µL (0.172 µg/µL, 39 µg/µL), 100 µL (0.174 µg/µL, 39 µg/µL) for AML II, III, IV and V, were mixed respectively with 250 µL, 300 µL, 160 µL, 50 µL and 100 µL of Hb-solution (1.8 µg Hb/µL in PBS). If necessary, additional PBS was added to reach final respective volumes of 350 µL, 550 µL, 330 µL, 190 µL and 240 µL before incubation at 4 °C overnight under gently mixing. 20 µL , 55 µL, 30 µL, 50 µL and 30 µL of a non-activated polyNIPAAm solution (100 µg/µL, in PBS) were added and gently mixed at 4 °C to get final total polyNIPAAm concentrations between 2 and 3 % w/v (values were respectively 2.0, 2.0, 2.0, 3.0 and 2.5 % w/v). After that a thermocycling step was done (precipitation at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). For washing, the Hb-AML complexes were redissolved at 4 °C in fresh PBS (respectively 370 µL, 605 µL, 360 µL,240 µL and 270 µL) following with a thermocycling step. A first elution step for removing low-affinity bound proteins was done by redissolving the Hb-AML complexes respectively in 370 µL, 605 µL, 360 µL, 240 µL and 270 µL of fresh 0.15 M NaCl, pH 11 following with a thermocycling step, recovering the supernatant and immediatly adjusting the pH to 8 with 1 M Tris-HCl, pH 7. For elution of the Hb, 370 µL, 605 µL, 360 µL, 240 µL and 270 µL of freshly prepared 5 M urea in 0.15 M NaCl, pH 11 were respectively used, following with a thermocycling step (removal of the AML), recovery of the supernatant and immediately adjusting of the pH to 8 with 1 M Tris-HCl, pH 7. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm, by the Drabkin test (conversion to cyanomethemoglobin) or by the NanoOrange test.

Table 4.2. Summary of the binding step for the affinity precipitation protocol with high pH elution. Solutions were prepared in binding buffer (PBS).

Binding step	AML I	AML II	AML III	AML IV	AML V
Hp (μg/ μL) in AML solution	1.82	0.33	0.3	0.172	0.174
polyNIPAAm (μg/ μL) in AML solution	50	24	40	39	39
AML solution vol (μL)	100	250	100	50	100
Hp (μg) in AML solution	182	82.5	30	8.6	17.4
polyNIPAAm (mg) in AML solution	5	6	4	2	4
Hb (μg/ μL)	1.8	1.8	1.8	1.8	1.8
Hb vol (μL)	250	300	160	50	100
Hb (μg)	450	540	288	90	180
Extra binding buffer (μL)	0	0	70	90	40
non-activated polyNIPAAm (mg)	2	5.5	3	5	3
non-activated polyNIPAAm solution vol (μL)	20	55	30	50	30
Final volume (μL)	370	605	360	240	270
% w/v total polyNIPAAm	2	2	2	3	2.5

For the low pH elution, initially the experiments followed the same steps as indicated above for the Hp-sepharose batch adsorption, see summary of the procedure in figure 4.1. Briefly, 100 μL of AML I-solution (1.82 μg Hp/μL, 50 μg polyNIPAAm /μL, in 0.1 M phosphate buffer pH 6.0), respectively 250 μL (0.33 μg/μL, 24 μg/μL), 100 μL (0.3 μg/μL, 40 μg/μL), 50 μL (0.172 μg/μL, 39 μg/μL), 100 μL (0.174 μg/μL, 39 μg/μL) for AML II to V, were mixed with 100 μL, 50 μL, 20 μL of Hb-solution (0.335 μg Hb/μL in 0.1 M phosphate buffer pH 6.0) respectively for AML I to III and with 25 μL, 50 μL of Hb-solution (0.067 μg Hb/μL in 0.1 M phosphate buffer pH 6.0) respectively for AML IV to V. A final volume of 200 μL, 300 μL, 190 μL, 165 μL and 190 μL was respectively reached before incubation at 4 °C overnight under gently mixing. 20 μL , 55 μL, 30 μL, 50 μL and 30 μL of a non-activated

polyNIPAAm solution (100 µg/µL, in 0.1 M phosphate buffer pH 6.0) were added and gently mixed in at 4 °C to get a final total polyNIPAAm concentrations of 3.2 % w/v. After that a thermocycling step was done (precipitation at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). For washing, the Hb-AML complexes were redissolved at 4 °C in fresh 0.1 M phosphate buffer pH 6.0 (respectively 220 µL, 355 µL, 220 µL, 215 µL and 220 µL) following with a thermocycling step. For elution of the Hb, the Hb-AML complexes were redissolved at 4 °C in 220 µL (respectively 360 µL, 220 µL, 220 µL and 220 µL) fresh 5% (v/v) formic acid in water (pH 2) followed by a thermocycling step. The Hb-containing supernatant was ajusted to pH 7 with 20 M NaOH. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm, by the Drabkin test (convertion to cyanomethemoglobin) or by the NanoOrange test.

Table 4.3. Summary of the binding step for the affinity precipitation protocol at low pH elution (without ammonium sulphate). Solutions were prepared in binding buffer (0.1 M phosphate buffer pH 6.0).

Binding step	AML I	AML II	AML III	AML IV	AML V
Hp (µg/ µL) in AML solution	1.82	0.33	0.3	0.172	0.174
polyNIPAAm (µg/ µL) in AML solution	50	24	40	39	39
AML solution vol (µL)	100	250	100	50	100
Hp (µg) in AML solution	182	82.5	30	8.6	17.4
polyNIPAAm (mg) in AML solution	5	6	4	2	4
Hb (µg/ µL)	0.335	0.335	0.335	0.067	0.067
Hb vol (µL)	100	50	20	25	50
Hb (µg)	33.5	16.8	6.7	1.68	3.4
Extra binding buffer (µL)	0	0	70	90	40
non-activated polyNIPAAm (mg)	2	5.5	3	5	3
non-activated polyNIPAAm solution vol (µL)	20	55	30	50	30
Final volume (µL)	220	355	220	215	220
% w/v total polyNIPAAm	3.2	3.2	3.2	3.2	3.2

In addition elution at low pH was combined with the addition of $(NH_4)_2SO_4$. Initially these experiments also followed the same steps as indicated above for the standard affinity precipitation (elution at low pH), figure 4.1, but this time ammonium sulphate was added during the thermocycling step (precipitation). Briefly, 100 µL of AML I - solution (1.82 µg Hp/µL, 50 µg polyNIPAAm /µL, in 0.1 M phosphate buffer pH 6.0), respectively 250 µL (0.33 µg/µL, 24 µg/µL), 150 µL (0.3 µg/µL, 40 µg/µL), 150 µL (0.172 µg/µL, 39 µg/µL), 100 µL (0.174 µg/µL, 39 µg/µL) for AML II to V, were mixed with 100 µL of Hb-solution (30 µg Hb/µL in 0.1 M phosphate buffer pH 6.0) for AML I and with 50 µL, 25 µL, 10 µL, 10 µL of Hb-solution (33.5 µg Hb/µL in 0.1 M phosphate buffer pH 6.0) respectively for AML II to V. If necessary, additionally 0.1 M phosphate buffer pH 6.0 was added to reach a final volume of

350 µL, 300 µL, 175 µL, 160 µL and 110 µL before incubation at 4 °C overnight under gently mixing. 150 µL, 100 µL, 15 µL, 5 µL and 5 µL of a non-activated polyNIPAAm solution (100 µg/µL, in 0.1 M phosphate buffer pH 6.0) were added and gently mixed in at 4 °C. After that a thermocycling step was done (1/4 vol (e.g., 125 µL for AML I) of a saturated $(NH_4)_2SO_4$ solution were added giving a final total polyNIPAAm concentration of 3.2 % w/v). For washing, the Hb-AML complexes were redissolved at 4 °C in respectively, 500 µL, 400 µL, 190 µL, 165 µL and 115 µL of fresh 0.1 M phosphate buffer pH 6.0 followed by a thermocycling step using 1/4 volume of a saturated ammonium sulphate solution. For elution of the Hb, the Hb-AML complexes were redissolved at 4 °C in 500 µL (respectively 400 µL, 190 µL, 160 µL and 110 µL) fresh 5% (v/v) formic acid in water (pH 2) followed by a thermocycling step (1/4 volume of a saturated ammonium sulphate solution was added). The Hb-containing supernatant was adjusted to pH 7 with 20 M NaOH. The Hb (oxyhemoglobin) concentration was determined by absorbance at 415 nm, by the Drabkin test (conversion to cyanomethemoglobin) or by the NanoOrange test.

Table 4.4. Summary of the binding step for the affinity precipitation protocol at low pH elution (with ammonium sulphate). Solutions were prepared in binding buffer (0.1 M phosphate buffer pH 6.0).

Binding step	AML I	AML II	AML III	AML IV	AML V
Hp (μg/ μL) in AML solution	1.82	0.33	0.3	0.172	0.174
polyNIPAAm (μg/ μL) in AML solution	50	24	40	39	39
AML solution vol (μL)	100	250	150	150	100
Hp (μg) in AML solution	182	82.5	45	25.8	17.4
polyNIPAAm (mg) in AML solution	5	6	6	6	4
Hb (μg/ μL)	30	33.5	33.5	33.5	33.5
Hb vol (μL)	100	50	25	10	10
Hb (mg)	3	1.7	0.8	0.3	0.3
Extra binding buffer (μL)	150	0	0	0	0
non-activated polyNIPAAm (mg)	15	10	1.5	0.5	0.5
non-activated polyNIPAAm solution vol (μL)	150	100	15	5	5
Total volume (μL)	500	400	190	165	115
saturated $(NH_4)_2SO_4$ solution (μL) = total volume / 4	125	100	47.5	41.3	28.8
Final total volume (μL)	625	500	237.5	206.3	143.8
% w/v total polyNIPAAm	3.2	3.2	3.2	3.2	3.2

4.3 Results and Discussion

4.3.1 Preparation and characterization of hemoglobin extracted from blood

Red blood cells were first separated from plasma and lysed by osmotic shock, causing the release of Hb and other cell components into the solution. Hb was partially purified by a two step fractionated ammonium sulphate precipitation. The first step was done at 20 % saturation of ammonium sulphate and 0 °C, where high Mw proteins were precipitated and eliminated from the solution. In a second step, the Hb-containing supernatant was further fractionated with 80 % ammonium sulphate saturation at 0 °C, where Hb was precipitated together with others middle Mw proteins, leaving low Mw proteins in solution. The Hb-containing pellet was recovered by centrifugation and resuspended and dialysed or gel filtrated for removal of the high amounts of salts originally present. The concentration of this partially purified Hb sample was determined by the Drabkin's test. The concentration before desalting was about 42 mg Hb/mL (0.65 mM). This value is below the normal concentration of Hb in blood (120-180 g/mL [3]), in fact the Hb recovery yield after release from red blood cells and precipitation with ammonium sulphate was ca. 25 %. After ammonium sulphate had been removed by dialysis, a final concentration of 30 mg Hb/mL (0.47 mM) was found. When the gel filtration (PD-10 column) was used for desalting, a final concentration of 35 mg Hb/mL (0.55 mM) was obtained.

As showed by Drabkin in figure 4.2, and also by van Kampen [26], derivatives from hemoglobin can be detected by spectrophotometric scanning of samples.

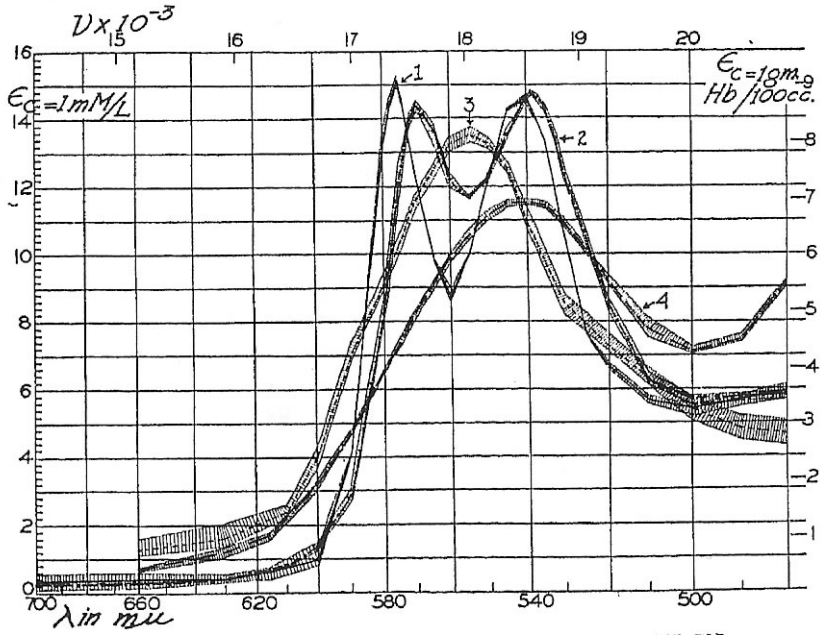


Figure 4.2. Source : Drabkin et al. [27]. Absorption curves of HbO_2 (curve 1), HbCO (curve 2), d-Hb (curve 3) and MHbCN (curve 4). Extinction coefficients are indicated per heme.

Spectrophotometric scans between 200-700 nm of the Hb sample used in this work confirmed the presence of HbO_2 (Figure 4.3).

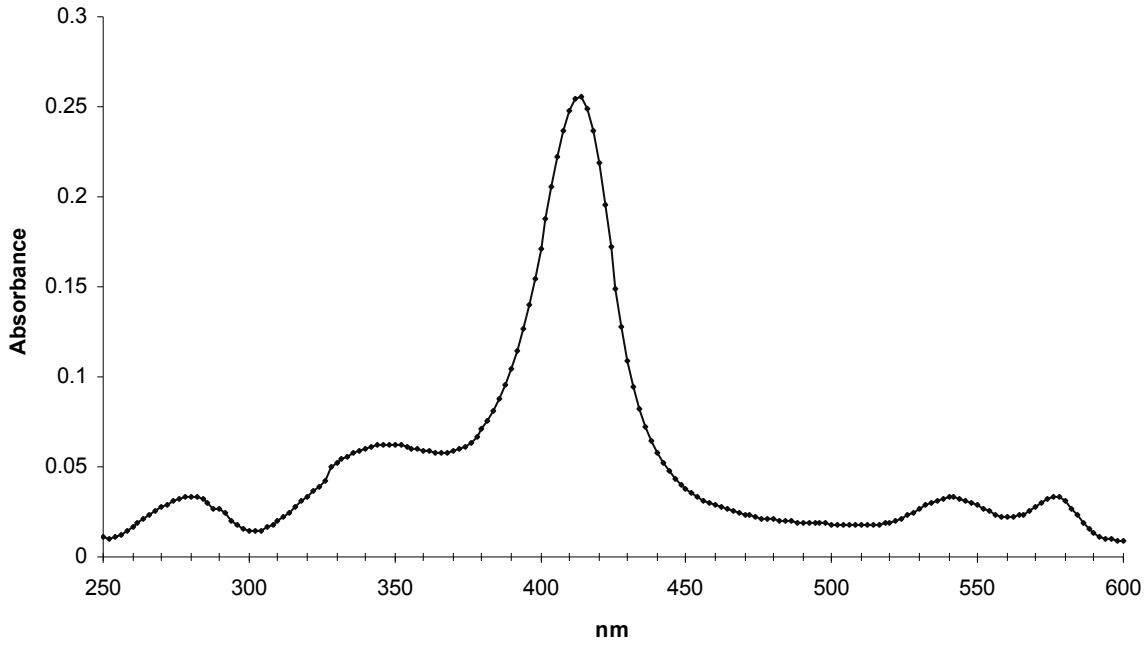


Figure 4.3. Scan specific to the oxyhemoglobin prepared in this work, with absorption peaks at 280, 415, 540 and 575 nm.

The best method for determination of hemoglobin concentrations is the Drabkin test, because all hemoglobins present in the sample are converted to cyanomethemoglobin prior to

measurement. In the cyanomethemoglobin method, potassium ferricynanide (an oxidising agent) converts the ferrous (Fe^{2+}) iron of hemoglobin to the ferric (Fe^{3+}) state (methemoglobin). This then combines with cyanide (CN^-) to form cyanmethemoglobin. This is a stable colored compound (i.e. unaffected by O_2 and CO_2 concentrations) and its measurement is not disturbed by any other element likely to be present in the sample or by turbidity [26]. Its concentration in solution can be estimated by its absorbance at 540 nm in a colorimeter / spectrophotometer. The problem is that the test is time consuming and that the KCN solutions are highly toxic. For this reason a second method was considered, which is faster and safer and which measures the absorbance at 415 nm of the oxyhemoglobin. To apply this method, it was necessary to assure that the Hb in the analysed sample was in the form of HbO_2. This was verified by scanning the Hb sample between 200-700 nm. When residual methemoglobin was detected, the reduction protocol was performed. Experimentally, during the Hb reduction protocol, a color change from red-brown (methemoglobin) to red-purple (ferrohemoglobin) was observed when the methemoglobin entered the dithionite zone (yellow zone). Oxygenation of the reduced Hb gave finally the desired oxyhemoglobin.

4.3.2 Synthesis and characterization of the AML

Synthesis and characterisation of the polyNIPAAm was done as already described on chapter 2. In order to aid process development, the CST of the polyNIPAAm, was recorded for 1 % (w/v) solutions in all relevent binding buffers (0.1 M phosphate buffer pH 6.0 or PBS (8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) pH 7.4), and elution solutions (5 % formic acid in Millipore water or 0.15 M NaCl, pH 11 or 5 M urea, 0.15 M NaCl, pH 11). The results are summarised in figure 4.4. The 0.15 M NaCl solution pH 11 (elution buffer for low-affinity bound proteins) gave a CST-value of around 32.5 °C. The CST-value in the binding buffers dropped to 31 °C in PBS and to 30 °C in the phosphate buffer. Most importantly, the CST was 26.5 °C in 5 M urea, 0.15 M NaCl, pH 11, while in 5 % formic acid / water the value was as low as 20.5 °C. Again these values were to be expected given the composition of these particular environments, but the values had to be taken into account when setting up the capture conditions, see below.

.

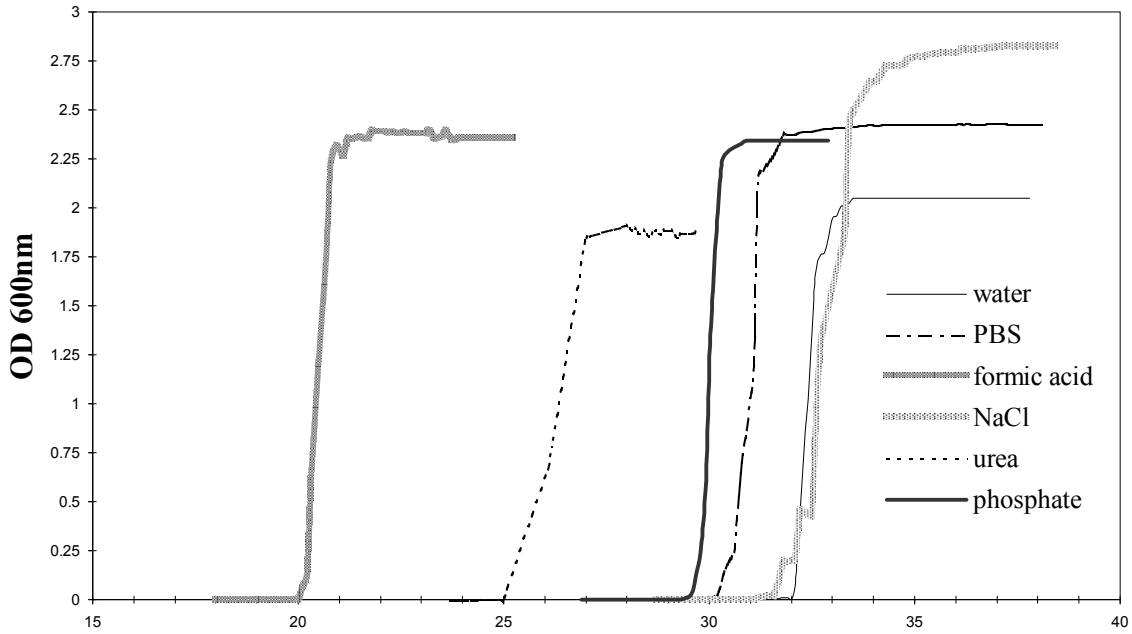


Figure 4.4. CST measurements of 1 % polyNIPAAm in solutions used for the affinity precipitation of hemoglobin

The carboxylic acid end group of the oligomeric polyNIPAAm was subsequently activated by NHS prior to coupling of Hp, as described previously. As verified spectrophotometrically, NHS-activation by the indicated protocol was successful for ca. 90 % of the end groups. This rate was taken into account when calculating the coupling ratios (see Table 4.5).

As the Hp sample from Sigma was of unknown phenotype composition, it was assumed to have a molar mass of 100'000 g/mol, as for type 1-1 Hp, i.e., assuming that large polymers are in relatively low abundance as indicated by Bowman [6]. The molar extinction coefficient of the Hp tetramer at 280 nm was estimated to 1.2 x 10^5 M^{-1} by measuring the absorbance of four known concentrations of Hp in solution. This value was confirmed in the literature [22, 40].

Different coupling ratios from 220 : 1 to 4 : 1 (polyNIPAAm to Hp) were investigated in this study (see Table 4.5). The tetrameric structure of the human Hp has about 60 primary amino groups (from lysine residues and the N-terminal amino acids) as found in the NCBI database [41]. Knowing that this value corresponds to the maximum number of sites on Hp able to react with the activated polyNIPAAm, molar ratios of polymer to coupling sites from 4 : 1 to 0.1 : 1 were calculated. In addition, polyNIPAAm concentrations of 3 % (w/v) were adjusted during the coupling. As indicated in Table 4.5, best results in terms of efficient coupling were

obtained with higher molar ratios between polyNIPAAm and Hp. In the case of AML I, almost all Hp was coupled to the polyNIPAAm, while in for AML IV and AML V only 1/5th of the Hp was coupled to the polyNIPAAm.

Table 4.5. Coupling ratios and yields

Coupling	AML I	AML II	AML III	AML IV	AML V
polyNIPAAm : haptoglobin (mol/mol)	220 : 1	100 : 1	50 : 1	10 : 1	4 : 1
polyNIPAAm : amino groups (mol/mol)	4 : 1	2 : 1	1 : 1	0.2 : 1	0.1 : 1
% haptoglobin conjugated to polyNIPAAm / total haptoglobin	91	87	40	20	20

It was subsequently verified that the NHS-activation of the polyNIPAAm did not significantly change the CST for a given buffer system. The AML showed identical precipitation / redissolution behavior as the non-activated polyNIPAAm in the subsequent affinity precipitations. The most consequential values for the set up of the affinity precipitation of the hemoglobin were therefore the CST in the elution buffers (5 % (v/v) formic acid in water (CST = 20.5 °C), 5 M urea in 0.15 M NaCl, pH 11 (CST = 26.5 °C)). In the subsequent affinity precipitation separations, a temperature of 4 °C was adjusted during all steps concerning resuspension of the AML pellet and all binding / dissociation steps of the affinity complex. During binding and washing steps, a precipitation temperature of 37 °C was used to accommodate the higher CST of the AML / affinity complex in this solution. All precipitation / centrifugation steps during elution were also done at 37 °C even if the temperature could potentially be lowered to 30-32 °C.

4.3.3 Affinity chromatography and batch adsorption results

Two established “binding / elution” procedures for affinity chromatography of Hb on Hp were found in the literature and, in order to maximise comparability, these protocols were

applied to the affinity precipitation, adding only the thermocycling step which is fundamental for the method. Binding conditions of the two methods were almost similar (buffer compositions, pH values), but the methods differed completely in regard to the elution approach, as one used a low pH (5 % formic acid, pH 2) and the other used a high pH buffer containing in addition a chaotropic agent (5 M urea, 0.15 M NaCl, pH 11). As indicated by Jayle [12] and Waks [25], the necessity for such harsh elutions conditions were to be expected as the binding between Hp and Hb is very strong and essentially irreversible over a wide pH range (4.4 to 10).

Following the protocol described above for the coupling of Hp to the sepharose column, 96 % of the initial amount of Hp could be coupled to the solid phase. To determine the coupling yield, it was first necessary to eliminate the NHS (released after coupling of Hp to the column) from the eluted sample containing the not-bound Hp, as this would have influenced the OD_{280} measurement. For this purpose PD-10 columns were used. Chromatography was done by gravity flow and the elimination of NHS was possible, allowing to use the absorbance measurement at 280 nm of Hp to determine the coupling efficiency.

Three affinity chromatography experiments with the high pH elution were done and an elution ratio of 0.09 mol Hb per mol of bound Hp was obtained. The results are summarized in table 4.6. Two affinity chromatography experiments with the low pH elution were done and an elution ratio of 0.015 mol Hb per mol of bound Hp was obtained. Tsapis [22] found a binding ratio of 0.39 mol Hb / mol Hp when Hp-sepharose columns (CNBr-activated sepharose-4B) was used to capture Hb by affinity chromatography. According to him, the difference observed between 0.39 mol Hb / mol Hp and the expected 1 : 1 ratio could be due either to Hp denaturation during coupling or to the fact that some Hp binding sites were hidden. The latter could occur during the Hp coupling due to the chemical binding and to the orientation of the Hp molecule on agarose. It has been determined that blocking of the amino groups of the Hp reduces the Hb-binding capacity suggesting that the amino groups may be involved in the Hb-Hp complex formation [10, 42]. Inhibition of the complex formation has also been observed when the amino groups of the Hb were blocked [12]. By comparison, Liau obtained an elution ratio of 0.75-1.00 mg Hp/mg Hb, using the high pH elution protocol, which corresponds to 0.48-0.64 mol Hp/mol Hb (= 1.5-2 mol Hb/mol Hp). It should be kept in mind, however, that in this case the Hp was in solution and the Hb was coupled to the solid phase (CNBr-activated sepharose-4B). It doesn't seem likely that the choice of NHS-activated

sepharose as solid phase could be responsible for the low yield obtained by the affinity chromatography. The manufacturer (Amersham) said that this solid phase combines the advantages of high stability and a spacer arm being suitable for coupling of all sizes of proteins, even for smaller proteins and peptides, having free amino groups.

Table 4.6. Summary of Affinity Chromatography and Batch Adsorption methods. The Hb / Hp molar ratio is compared at different steps of the purification (initial, binding, and elution).

Method used and comments		mol Hb / mol Hp		
		Initial ratio	Binding ratio	Elution ratio
Theoretical affinity	Hp-binding experimets with α and/or β chains of Hb [12, 18, 22]	> 1 < 1	1 < 1	
Affinity Chromatography	Hb-column for Hp purification (Liau et al. [29])	3.4 - 5.1	1.5 - 2	
	Hp-column for Hb purification (Tsapis et al. [22])	0.25 - 20	0.39	
	Hp-column for Hb purification (Tsapis et al. [38])	0.03 - 0.6	0.9	
	Hp-column for Hb purification High pH elution protocol	115		0.09
	Hp-column for Hb purification Low pH elution protocol	115		0.015
Batch Adsorption	Hp-column for Hb purification High pH elution protocol	15		0.04
	Hp-column for Hb purification Low pH elution protocol	15		0.02

In addition to the affinity precipitation chromatography experiments, the Hp-Sepharose material was also used in loose form for affinity adsorption experiments mimicking affinity

precipitation conditions. In particular, the affinity precipitations conditions were simulated / mimicked (mixing, thermoprecipitation and centrifugation steps) using the Hp-sepharose in suspension for the Batch Adsorption method (table 4.6). In two tests, on the average 0.04 mol Hb/mol Hp were recovered by the high pH elution protocol. For the low pH elution protocol 0.02 mol Hb/mol Hp were recovered on the average in two experiments.

Considering the affinity chromatography and the batch adsorption methods, it can be concluded that both elution protocols give a low elution ratio. Nevertheless, it seems that the high pH elution protocol is the better one of the two protocols used. Studies have indicated that if the pH of the buffer is below 4.4 or if urea is used, the binding capacity of the Hp is rapidly lost, but also that Hp may be heated up to 53 °C for 30 min without any demostrable loss of binding capacity for Hb [11, 23]. The irreversible binding of Hb to Hp is an advantage as far as the total recovery of Hb from the hemolysates, and thus its complete purification, is concerned. However, the absence of dissociation of the Hb-Hp complexes raised the problem of the recovery of the purified Hb. Tsapis et al. proposed to remove first the heme group from the formed Hp-Hb complex by the acetone-HCl method and then do the low pH elution [38]. In this case the reaction between the apohemoglobin (Hb without heme) and the Hp is reversible.

4.3.4 Isolation of hemoglobin by haptoglobin-polyNIPAAm affinity precipitation.

The goal of this investigation was an evaluation of the comparative worth of affinity precipitation in the purification of Hb from human blood. The basic procedures are outlined in figure 4.1. Based on the AML characterization results and on works cited [17, 29, 30, 36-39], the affinity precipitation experiments were set-up. The Hb was then captured by the Hp conjugated polyNIPAAm (affinity precipitation with the five AML constructed). The results were compared with affinity chromatography and affinity batch adsorption in terms of yield. Affinity precipitations in this chapter were all done at final polyNIPAAm concentrations between 2 and 3.2 % (w/v) to assure abrupt and quantitative precipitation. In order to reduce the amount of expensive AML used in the experiments to that required for capturing the target molecule, the final polyNIPAAm concentration was provided via the addition of non-activated polyNIPAAm.

The first experiments were done with the low pH elution protocol. 3.6×10^{-6} mmol (0.23 mg) Hb were recovered using 1.8×10^{-6} mmol Hp bioconjugate (AML I), giving an elution ratio of about 2 mol Hb/mol Hp. As indicated in table 4.7, all experiments done with a molar excess of Hb compared to the Hp-containing AML and independent of the protocol followed, gave an elution ratio between 1.1-2.7 mol Hb/mol Hp, i.e., similar values as found by Liau in binding assays. If the molar Hb/Hp ratio was lower than 1, the elution ratio was also smaller than 1 as determined by Tsapis et al. (binding curves of Hb to Hp-sepharose in ref. [22]). Particularly in my case, five experiments were done with an initial molar ratio of 0.3 mol Hb per mol Hp, giving after binding an elution ratio of 0.24 mol Hb per mol Hp.

Table 4.7. Summary of the affinity precipitation assays

AML and protocol used		mol Hb / mol Hp	
	mmol Hp	Before binding	After elution
AML I			
with $(NH_4)_2SO_4$, low pH elution	1.82E-06	25	2.04
without $(NH_4)_2SO_4$, low pH elution	1.82E-06	0.285	0.24
without $(NH_4)_2SO_4$, high pH elution	1.82E-06	3.830	1.11
AML II			
without $(NH_4)_2SO_4$, low pH elution	8.29E-07	0.313	0.26
without $(NH_4)_2SO_4$, high pH elution	8.29E-07	10.105	1.40
AML III			
without $(NH_4)_2SO_4$, low pH elution	3.00E-07	0.348	0.25
without $(NH_4)_2SO_4$, high pH elution	3.00E-07	14.880	2.75
AML IV			
without $(NH_4)_2SO_4$, low pH elution	8.60E-08	0.303	0.24
without $(NH_4)_2SO_4$, high pH elution	8.60E-08	16.230	2.71
AML V			
without $(NH_4)_2SO_4$, low pH elution	1.74E-07	0.298	0.23
without $(NH_4)_2SO_4$, high pH elution	1.74E-07	15.990	2.46

The results indicated in table 4.7 were obtained with five AML, different molar amounts of Hp were also used. To determine the purification capacity of the AML (Hp-polyNIPAAm), a normalization was done from these experimental results using the lowest molar amount of Hp present in one of the AML (AML IV, 8.6×10^{-8} mmol) and its final average volume (227 μL) as normalization references. (see table 4.8 for calculations).

Table 4.8. Normalization of results shown in table 4.7. Example done with the AML I (without ammonium sulphate, high pH elution) to obtain the r and c values used for the Scatchard plot.

	obtained experimentally	after normalization
Hp in AML I (mmol)	1.82×10^{-6}	**8.6×10^{-8}**
final volume (μL)	370	**227**
Binding ratio (mol Hb/mol Hp)	3.83	3.83
Hb initially added (μM)	18.8	1.45
Elution ratio (mol Hb/mol Hp)	1.11	1.11 = ***r***
Hb initially added (mmol)	6.97×10^{-6}	3.29×10^{-7}
Hb bound (mmol)	2.02×10^{-6}	9.55×10^{-8}
Hb free or not bound (mmol)	4.95×10^{-6}	2.34×10^{-7}
Hb free or not bound (μM)	13.4	1.03 = ***c***

After this normalization, a "saturation" curve was determined using the elution ratio (instead of a binding ratio) and the initial-adsorbed Hb concentration, assuming that the real amount of Hb bound to the Hp is at least the amount of eluted Hb (figure 4.5). A Scatchard plot was constructed to determine the number of Hb molecules that were bound per Hp molecule under these conditions (figure 4.6). The Scatchard relation [43] is:

$$\frac{r}{c} = K\,n - K\,r$$

where r represents the number of Hb molecules bound per Hp molecule at c, free concentration of Hb (Hb not bound to the AML), n is the maximum number of Hb molecules that can be bound per Hp molecule and K is the association constant.

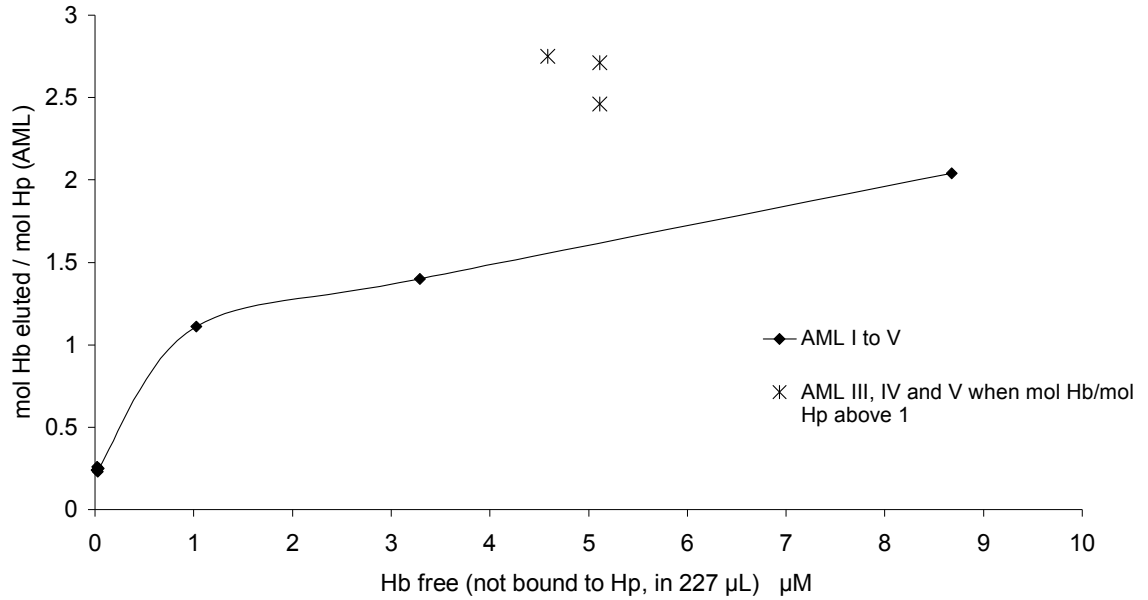


Figure 4.5. Normalized adsorption curve. Increasing amounts of hemoglobin added to 86 pmol Hp (final volume 227 µl). See table 4.8 for an example of normalization of experimental results from table 4.7. The three highest elution ratio (2.46, 2.71 and 2.75 mol Hb/mol Hp) obtained when using between 14.8-16.0 mol Hb/mol Hp (initial ratio), weren't considered.

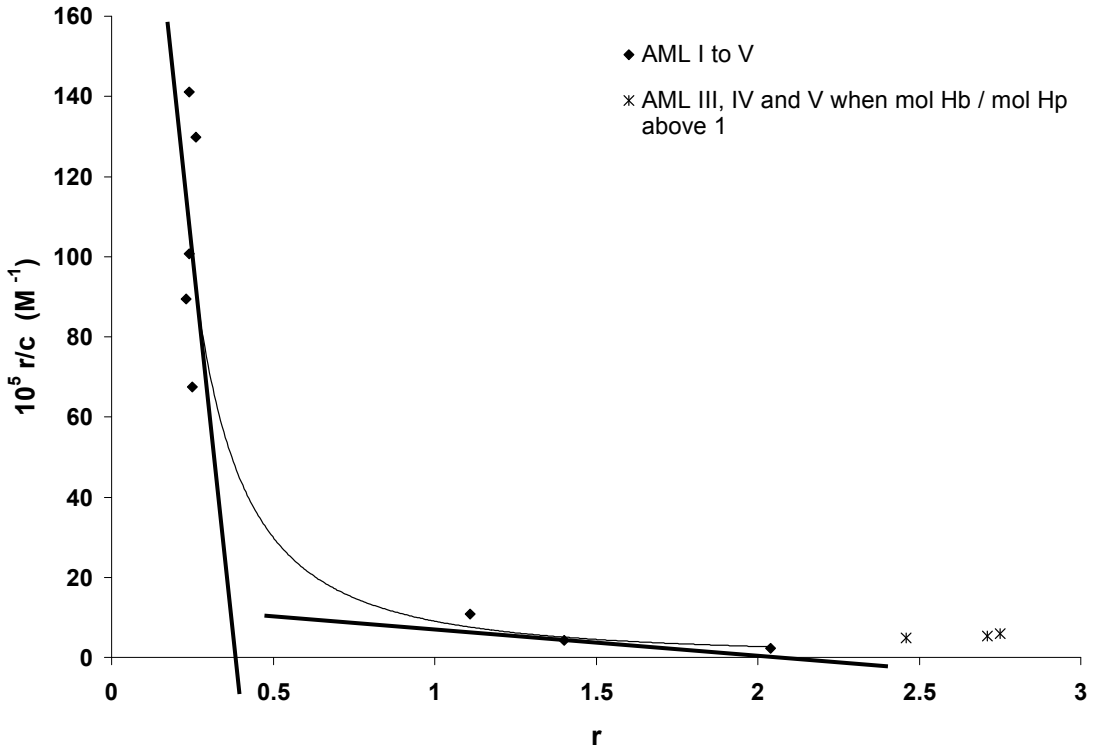


Figure 4.6. Scatchard plot of hemoglobin binding on haptoglobin (AML). r and c values were determined as indicated in table 4.8 (normalization). Elution ratios of 2.46, 2.71 and 2.75 mol Hb/mol Hp weren't considered.

In figure 4.5, it is shown that a maximum of 1.5-2 molecules of Hb can be bound per Hp (AML), which confirms the results obtained by Liau et al. [29]. On the other hand, in figure 4.6, the shape of the Scatchard plot indicates a heterogeneity of binding sites in the Hp (AML). A minor number of Hb molecules were bound with high-affinity (estimated higher than 10^7 M^{-1}) and a major one with a low-affinity constant (estimated around 10^5 M^{-1}). By extrapolating to the r axis, it was estimated that the maximum number *n* of Hb molecules bound per Hp molecule (AML) was around 2 and the number of Hb molecules bound per Hp molecule (AML) with high-affinity was around 0.3. It was found on the literature that the maximum number of Hb molecules bound to the Hp is 1, as described before in table 4.6, nevertheless the value obtained experimentally confirms the Liau et al. results. The value of 0.3 mol Hb/mol Hp on the other hand corresponds to the values determined in binding assays by Tsapis [22].

Most of the experiments previously done to determine the maximum number of binding sites for Hb in Hp were realised with isolated α and/or β chains (dissociated Hb molecules). Tsapis et al., e.g. his experiments with α chains (instead of Hb tetramers), determined a maximum of 4.3 α chains bound per molecule of Hp (4 binding sites in the Hp), from which 1.5 α chains showed a high affinity association constant for Hp (around 5 x 10^5 M^{-1}). Tsapis et al. also found that the major population of sites had a low-affinity constant of around 6 x 10^4 M^{-1}. As the α chains show a much lower affinity for Hp than the Hb tetramer [18], the affinity association constant of the Hp (AML), estimated to be higher than 10^7 M^{-1}, seems to be in the expected range as it's above the α chains association constant found by Tsapis, but also near to 10^{15} M^{-1} (i.e. the value found in ref. [24]).

Results from table 4.5, table 4.7, figure 4.5, and figure 4.6 were analysed to determine the influence of the polyNIPAAm/haptoglobin coupling ratio, on the elution ratio obtained by the different AML. It can be observed that AML III, IV and V behave differently from the other AML when the initial molar ratio of Hb/Hp is higher than 1. Their high elution ratio may be related to the low polyNIPAAm/Hp ratio used during coupling and may be caused by a better exposition of binding sites of the Hp-ligand in these AML to the Hb.

MS analysis of samples obtained before binding and after elution was done to confirm the presence of intact Hb. When comparing figure 4.7 (Hb prior to binding to the Hp (AML)) to

figure 4.8 (Hb eluted from the Hb-AML complex), it is obvious that the affinity precipitation reached the goal of purifying intact Hb.

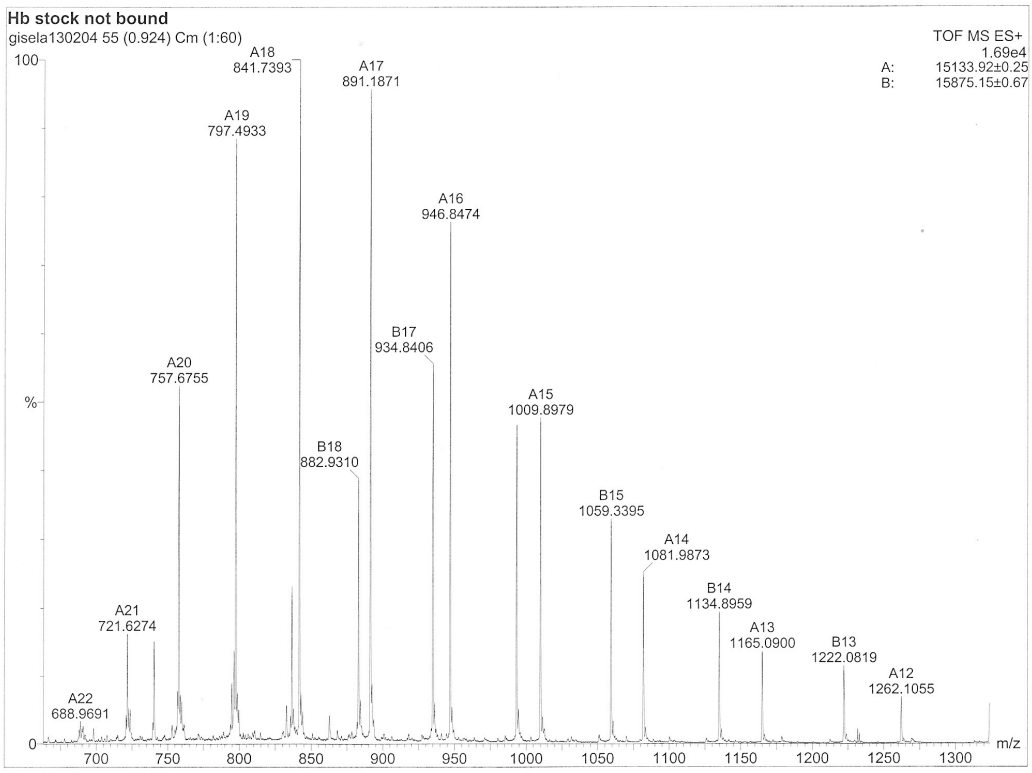


Figure 4.7. MS analysis of the Hb not bound to the Hp in the PBS binding step (high pH elution protocol, affinity precipitation). Preliminary gel filtration with deoinized water was necessary before analysis.

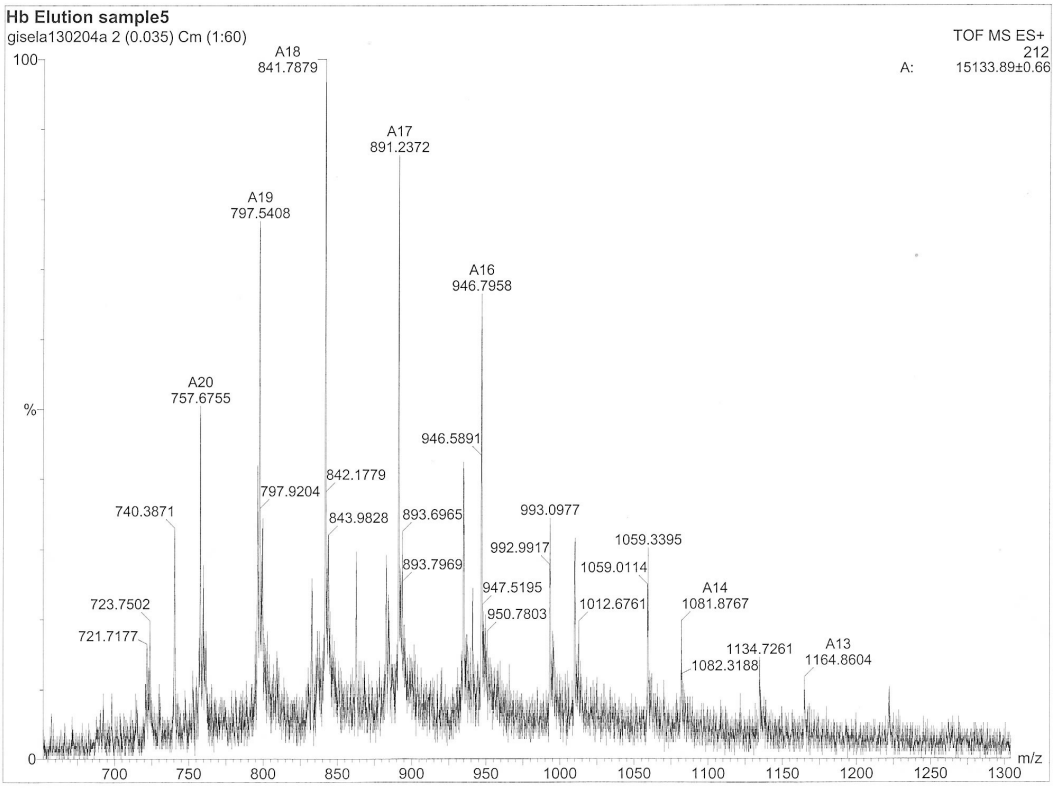


Figure 4.8. MS analysis of the sample eluted with 5 M urea, 0.15 M NaCl, pH 11 solution (high pH elution protocol, affinity precipitation). Preliminary gel filtration with deoinized water was necessary before analysis.

Beisembaeva studied the effects of pH, urea and sulphate ions on the hemoglobin-binding capacity of Hp of sheep and on the properties of the Hb-Hp complex [37]. It was found that the optimal conditions for the Hp-Hb complex formation are pH values within the range of 4.5-7.0. This infomation confirms the choice of binding buffers used for Hb purification. Additionally, Beisembaeva showed that 6 M urea and 0.1 M sulphate ions, including $(NH_4)_2SO_4$, produce an inactivating effect on the formation and on the stability of the complex. This is probably due to the interaction of these compounds with the Hp molecule, which changes Hp reactivity and the properties of the Hb-Hp complex. The high pH elution buffer used on this project has an urea concentration of 5 M, i.e., lower than the value studied by Beisembaeva. However, in the case of ammonium sulphate, the thermocycling step used a 0.8 M concentration (a saturated solution of 4 M was diluted 5 times) i.e., 8 times higher than the studied value, which may have had an influence on the Hb-Hp complex formation. Even if Beisembaeva used sheep Hp instead of human Hp, it should be mentioned that Hp isolated from most nonhuman species resembles human Hp1-1 on gel electrophoresis and appears as a

single component [6]. Based on these information, it was decided to perform affinity precipitation assays without using ammonium sulphate in the thermocycling step (especially during the binding step), in order to avoid any negative effect on the binding and stability of the Hp-Hb complex.

Table 4.7 and table 4.9 indicate an elution ratio of 2 when the AML I was used with ammonium sulphate, while values between 1.1 and 2.7 were obtained when the affinity precipitation was done without ammonium sulphate. Table 4.9 shows results obtained with "fresh" samples of AML II and III and with recycled AML I to III, after the low pH elution protocol. It can be seen that the elution ratio is very low in some of these cases, indicating that something has influenced the complex formation and / or its stability. The affinity precipitation with "fresh" AML was done by adding ammonium sulphate in the thermocycling steps, and as the elution ratio was so low, it was decided to do further affinity precipitations without ammonium sulphate. As the elution ratio of the recycled AML were low, without adding ammonium sulphate, a second reason for the low elution ratio could be the use of the low pH elution solution (pH 2) as explained above. The results summarized in table 4.9 show the possibility of recycling the AML for further purifications. The residual binding (elution) efficiency of the Hp bioconjugated was tested after two or three recycling steps, and it was found a reduction of 37 to 97 % of the AML's elution ratio from one assay to the next one. The recycling of the AML was thus not possible, probably because of the harsh elution conditions necessary for the dissociation of the Hb-Hp complex. As the concentration of Hb in the eluted fractions was low, it was difficult to determine their exact value. The amount of Hb purified by affinity precipitation in some cases couldn't be quantified by absorbance spectroscopy (lower detection limit of 1 μg/mL), that's why then the NanoOrange Assay was used to determine these values (detection limit: 10 ng/mL).

Table 4.9. Analysis of stability and recycling possibilities of the Hp bioconjugate under harsh elution protocol conditions.

		Elution ratio (mol Hb / mol Hp)		
AML	elution protocol	1st assay	2nd assay	3rd assay
AML I	low pH	2.0 (+)	0.0438 (+)	0.0275 (-)
AML II	low pH	0.0138 (+)	0.00403 (-)	n.d.
AML III	low pH	0.0427 (+)	0.00593 (-)	n.d.
AML I	high pH	1.11 (-)	0.0966 (-)	n.d.
AML II	high pH	1.40 (-)	0.5706 (-)	n.d.
AML III	high pH	2.75 (-)	0.6103 (-)	n.d.
AML IV	high pH	2.71 (-)	0.5509 (-)	n.d.
AML V	high pH	2.46 (-)	0.2326 (-)	n.d.

(+) = with ammonium sulphate (-) = without ammonium sulphate

n.d. : not done

Comparison of the elution ratio using the high pH elution protocol (without adding ammonium sulphate) between “fresh” AML I to V and recycled AML I to V in table 4.9, shows that the elution ratio is also reduced, but not as much as it was in case of the low elution protocol. These results indicate that the 5 M urea causes less damage than the 5 % formic acid for the Hp-ligand. It was also determined that the molar ratios of Hb bound / Hp

obtained were within the expected range i.e. as found previously by Liau and Tsapis., or even increased.

These experiments also indicated that the presence of ammonium sulphate during the thermocycling steps didn't give a better recovery of the Hb. One reason for the addition of ammonium sulphate during the thermocycling step (precipitation) was the favourable morphology of the flocks and in consequence the more quantitative precipitation. The quality of the pellet during the thermoprecipitation and centrifugation steps was evaluated when no ammonium sulphate was introduced to the solution. Parameters like the shape, the aggregation and the position of the pellet were checked visually to assure that the AML was not lost and that the protocol was not negatively affected. No important change in the pellet parameters was observed when the affinity precipitation was done without ammonium sulphate. For that reason ammonium sulphate was removed from the protocol. Two different concentrations of non-activated polyNIPAAm (3 % and 4 %) in 0.1 M phosphate buffer, pH 6 were also analysed by thermoprecipitation and it was confirmed that within this range, the precipitation behaviour is conserved.

Additionnally experiments should be done to analyse the results obtained here. It seems that an optimum AML should be situated between AML I and AML III, combining a optimum polyNIPAAm / Hp ratio (between 200 and 50), without blocking important binding sites for Hb on the Hp but still having enough attached polyNIPAAm to allow for a good recovery of the AML. About the protocol used, it is proposed to use a molar excess of Hb over Hp and to apply the high pH elution protocol without adding ammonium sulphate in the thermocycling step, to avoid affecting the formation and the stability of the complex. 5 M urea, 0.15 M NaCl, pH 11 was less denaturing than 5 % formic acid / water (pH 2), but still strong enough to be used for the dissociation of the complex. If the AML should be recovered and recycled, as it is desirable when expensive ligands are used, additionally affinity precipitation tests should be done to find the optimum urea concentration necessary for conservation of the binding capacity.

4.4 Conclusions

Affinity precipitation was included as the final purification step in a hemoglobin isolation protocol from blood. The first steps on the processes were realized by traditional methods

(lyses of red blood cells and precipitation with ammonium sulphate). Five different AML (Hp-polyNIPAAm) were constructed changing the polymer : haptoglobin coupling ratio. Affinity precipitation was compared to affinity chromatography and batch adsorption methods using two binding / elution protocols. The harsh elution conditions (one at pH 2, the other with 5 M urea at pH 11) needed for dissociation of the Hb-Hp complex rendered the recycling of the AML difficult. The ammonium sulphate normally added during the precipitation of the Hb-AML complexes was take out of the protocol to avoid negative effects. The affinity precipitation is a suitable method for purification of hemoglobin as the results obtained with the AML confirm the values obtained by others using current methods in terms of the binding ratio between haptoglobin and hemoglobin and the association constant.

Affinity precipitation is thus a viable alternative to chromatography-based purification of hemoglobin. The AML can be used directly to purified the Hb from a partially purified Hb sample. Even if it wasn't study here, the removal of debris of red cells and proteins others that Hb should be possible in the presence of the AML, without doing the preliminary ammonium sulphate precipitation step, as long as the temperature of the binding step for Hb-Hp complex formation is below the CST .

4.5 References

1. Hemoglobin, in Merck Index, 12th, S. Budavari, Editor. **1996**. 794, # 4682.
2. Sigma-Aldrich. *Product information sheet for hemoglobin*. 1996 Available from: http://www.sigmaaldrich.com/sigma/product%20information%20sheet/h2500pis.pdf.
3. *Hémoglobine*. Available from: http://www.vulgaris-medical.com/encyclopedie/hemoglobine-2241.html.
4. Giblett, E.R., Hemoglobin, in Genetic markers in human blood. **1969**, Blackwell Scientific: Oxford. 346-423.
5. Giblett, E.R., Haptoglobin, in Genetic markers in human blood. **1969**, Blackwell Scientific: Oxford. 63-125.
6. Bowman, B.H.; Kurosky, A., Haptoglobin: The evolutionary product of duplication, unequal crossing over, and point mutation, in Advances in human genetics, H. Harris and K. Hirschhorn, Editors. **1982**, 12, Plenum Press: New York. 189-261.
7. Sigma-Aldrich. *Haptoglobin, Product Information*. 2006 Available from: http://www.sigmaaldrich.com/sigma/datasheet/h3536dat.pdf.
8. Smithies, O. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem. J., **1955**, 61, 629-641.
9. Ibelgaufts, H. *Haptoglobin*. 2002 Available from: http://www.copewithcytokines.de/cope.cgi?key=Haptoglobin.
10. Putnam, F.W., Haptoglobin, in The plasma proteins: Structure, function and genetic control, 2nd, F.W. Putnam, Editor. **1975**, 2, Academic Press, Inc.: Orlando. 1-50.
11. Laurell, C.B.; Grönvall, C. Haptoglobins. Adv. Clin. Chem., **1962**, 5, 135-172.
12. Jayle, M.F.; Moretti, J. Haptoglobin: Biochemical, genetic and physiopathologic aspects. Progr. Hematol., **1962**, 3, 342-359.
13. Nagel, R.L.; Gibson, Q.H. Kinetics and mechanism of complex formation between hemoglobin and haptoglobin. J. Biol. Chem., **1967**, 242, 3428-3434.
14. Nagel, R.L.; Wittenberg, J.B.; Ranney, H.M. Oxygen equilibria of the hemoglobin-haptoglobin complex. Biochim. Biophys. Acta, **1965**, 100, 286-289.
15. Balla, J.; Jacob, H.S.; Balla, G.; Nath, K.; Eaton, J.W. Endothelial-cell heme uptake from heme proteins: Induction of sensitization and desensitization to oxidant damage. Proc. Natl. Acad. Sci. USA, **1993**, 90, 9285-9289.
16. Nagel, R.L.; Rothman, M.C.; Bradley, T.B., Jr.; Ranney, H.M. Comparative haptoglobin binding properties of oxyhemoglobin and deoxyhemoglobin. J. Biol. Chem., **1965**, 240, PC 4543-4545.
17. Hensley, P.; Edelstein, S.J.; Wharton, D.C.; Gibson, Q.H. Conformation and spin state in methemoglobin. J. Biol. Chem., **1975**, 250, 952-960.
18. Chiancone, E.; Alfsen, A.; Ioppolo, C.; Vecchini, P.; Finazzi Agro, A.; Wyman, J.; Antonini, E. Studies on the reaction of haptoglobin with haemoglobin and haemoglobin chains. J. Mol. Biol., **1968**, 34, 347-356.
19. Hinton, R.H.; Dobrota, M.; Mullock, B.M. Haptoglobin-mediated transfer of haemoglobin from serum into bile. FEBS Letters, **1980**, 112, 247-250.
20. Kino, K.; Tsunoo, H.; Higa, Y.; Takami, M.; Hamaguchi, H.; Nakajima, H. Hemoglobin-haptoglobin receptor in rat liver plasma membrane. J. Biol. Chem., **1980**, 255, 9616-9620.
21. Kazim, A.L.; Atassi, M.Z. Haemoglobin binding with haptoglobin. Biochem. J., **1981**, 197, 507-510.

22. Tsapis, A.; Rogard, M.; Alfsen, A.; Mihaesco, C. Binding of human hemoglobin and its polypeptide chains with haptoglobin coupled to an agarose matrix. Eur. J. Biochem., **1976**, 64, 369-372.
23. Gordon, S.; Bearn, A.G. Hemoglobin binding capacity of isolated haptoglobin polypeptide chains. Proc. Soc. Exp. Biol. Med., **1966**, 121, 846-850.
24. Hwang, P.K.; Greer, J. Interaction between hemoglobin subunits in the hemoglobin-haptoglobin complex. J. Biol. Chem., **1980**, 255, 3038-3041.
25. Waks, M.; Alfsen, A.; Schwaiger, S.; Mayer, A. Structural studies of haptoglobin. III. Interactions with hemoglobin. Arch. Biochem. Biophys., **1969**, 132, 268-278.
26. van Kampen, E.J.; Zijlstra, W.G. Determination of hemoglobin and its derivatives. Adv. Clin. Chem., **1965**, 8, 141-187.
27. Drabkin, D.L.; Austin, J.H. Spectrophotometric studies II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. J. Biol. Chem., **1935**, 112, 51-65.
28. Amersham. *Instructions for PD-10 Desalting column*. Available from: http://www4.gelifesciences.com/aptrix/upp00919.nsf/Content/BF18004B78A8CE78C12570C4000DCB31/$file/52130800AR.pdf.
29. Liau, C.Y.; Chang, T.M.; Pan, J.P.; Chen, W.L.; Mao, S.J.T. Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. J. Chromatogr. B, **2003**, 790, 209-216.
30. Tsuruga, M.; Matsuoka, A.; Hachimori, A.; Sugawara, Y.; Shikama, K. The molecular mechanism of autoxidation for human oxyhemoglobin. J. Biol. Chem., **1998**, 273, 8607-8615.
31. Dixon, H.B.F.; McIntosh, R. Haematology. Reduction of methaemoglobin in haemoglobin samples using gel filtration for continous removal of reaction products. Nature, **1967**, 213, 399-400.
32. Stocker, G.; Vandevyver, C.; Hilbrig, F.; Freitag, R. Purification of RT-PCR competent poly(A) mRNA from crude cell lysate by affinity precipitation. Biotechnol. Prog., **2006**, 22, 1621-1629.
33. Garret-Flaudy, F. Thermoresponsive oligomers - solubility and applications for affinity precipitation, **2000**, PhD Thesis EPFL, Lausanne
34. Chen, G.; Hoffman, A.S. Preparation and properties of thermoreversible, phase-separating enzyme-oligo(*N*-isopropylacrylamide) conjugates. Bioconjugate Chem., **1993**, 4, 509-514.
35. Amersham. *Instructions for NHS-Activated Sepharose 4 Fast Flow*. Available from: http://www5.gelifesciences.com/aptrix/upp00919.nsf/Content/B560C841A63B1352C1256ECC000849DC/$file/71500014AC.pdf.
36. Shim, B.S.; Jue, D.M. Simple spectrophotometric determination of haptoglobin-haemoglobin complex in haemolysed samples. Scand. J. Clin. Lab. Invest., **1986**, 46, 45-51.
37. Beisembaeva, R.U. Effects of various factors on the formation and properties of sheep haptoglobin-hemoglobin complexes. Biokhimiia, **1982**, 47, 767-772.
38. Tsapis, A.; Hinard, N.; Testa, U.; Dubart, A.; Vainchenker, W.; Rouyer-Fessard, P.; Beuzard, Y.; Rosa, J. Globin-chain affinity chromatography on sepharose-haptoglobin: a new method of study of hemoglobin synthesis in reticulocytes, in bone marrow and in colonies of erythroid precursors. Eur. J. Biochem., **1980**, 112, 513-519.
39. Asakawa, J.; Kodaira, M.; Nakamura, N.; Satoh, C.; Fujita, M. Chimerism in humans after intragenic recombination at the haptoglobin locus during early embryogenesis. Proc. Natl. Acad. Sci. USA, **1999**, 96, 10314-10319.

40. Hermann-Boussier, G.; Moretti, J.; Jayle, M.F. Etude de l'haptoglobine. 2. Propriétés physiques et chimiques des haptoglobines des types I et II et de leur complexes avec l'hémoglobine. Bull. Soc. chim. Biol., **1960**, 42, 837-852.
41. *Human haptoglobin*. Available from: http://www.ncbi.nlm.nih.gov/.
42. Shinoda, T. The role of amino groups in the formation of hemoglobin-haptoglobin complex. J. Biochem., **1965**, 57, 100-102.
43. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., **1949**, 51, 660-672.

5. Affinity precipitation for antibody purification

5.1 Introduction

Antibodies have a wide application range, including research, diagnostics, and more recently also therapeutics [1, 2]. Depending on the intended use, the antibody needs to be produced in sufficient quantity and purity. Especially for therapeutic applications, this may pose quite a challenge for the downstream process [3, 4]. Typically, the first step in an antibody purification scheme is a capture from the dilute process stream, using some kind of biospecific affinity interaction [5]. Chromatographic (affinity) methods are widely used, but are expensive and difficult to scale up. As an alternative, affinity precipitation is proposed especially for large scale antibody purification. In this chapter, polyNIPAAm was conjugated to Protein A (Protein A affinity precipitation), to mimics thereof, or to the FLAG peptide (Antigen affinity precipitation), leading to the construction of different AML subsequently used for the isolation of the monoclonal M1-antibody IgG (also named anti-FLAG or 4E11), as target molecule. The demand for mAb is steadily increasing due to their large potential in therapeutic and diagnostic applications [5]. Therefore monoclonals are a very good example of a bulk product, which has to be purified efficiently. The Protein A affinity precipitation is a general approach for antibody purification, while the antigen affinity precipitation is a specific approach to the purification of IgG 4E11.

Antibodies or immunoglobulins are large Y-shaped glycoproteins. In humans, there are five antibody isotypes or classes (IgM, IgD, IgG, IgA and IgE). This chapter will focus on the affinity precipitation of an antibody that belongs to the IgG class. Immunoglobulin G (IgG) provides the majority of antibody-based immunity against invading pathogens [6]. It's the most abundant immunoglobulin class and is approximately equally distributed between blood and tissue liquids, constituting 75 % of serum immunoglobulins in humans [7]. IgG are used by the immune system to identify and neutralize foreign elements called antigens. IgG has an approximate molecular weight of 150 kDa. The non-antigen binding domain of an IgG is known as the constant region or Fc (Fragment, crystallizable) region, and has a cell receptor function, which is independent of the antigen binding domain and function (figure 5.1). In humans there are four subclass of IgG: IgG_1, IgG_2, IgG_3 and IgG_4. The Fc region is the same for all immunoglobulins of the same subclass but differs between subclasses [8]. IgGs have

two identical disulfide-linked, antigen combining sites known as the Fab domains, which contain a highly variable sequence to provide antigen binding specificity [9, 10].

Antibodies reversibly bind to their antigens by non-covalent interactions; they use hydrogen bonds, Van der Waals forces, or electrostatic forces. Depending on the structure of the antibody, which varies with isotype, and the structure of the antigen, an antibody may have either one (monovalent) binding interaction with an antigen, or multiple simultaneous (multivalent) interactions [11].

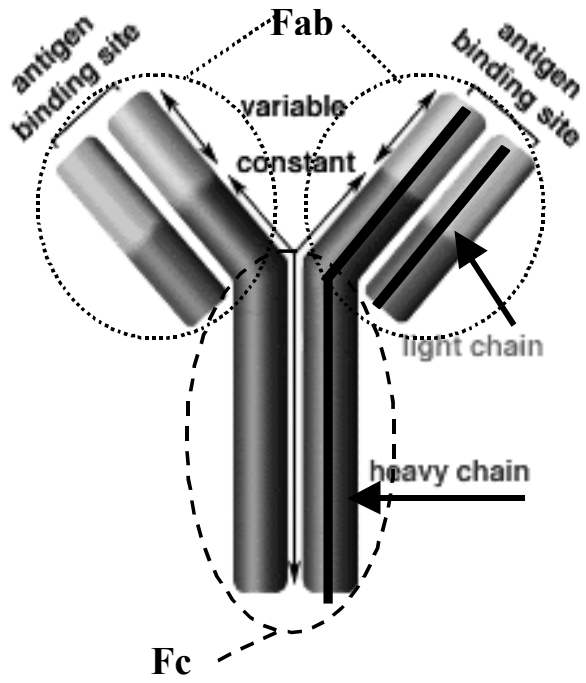


Figure 5.1. Structure of immunoglobulin G (IgG). Adapted from ref. [12]

Protein A consist of a single polypeptide chain with a Mw around 45 kDa and contains little or no carbohydrate (Reviewed in ref [13, 14]). It can be isolated from the cell wall of most strains of *Staphylococcus aureus*. The native molecule is composed of about 50 % α-helices. Amino acid analysis shows that the protein has a C-terminal lysine and a blocked N-terminal amino acid. As primary amino groups are important for the coupling of affinity ligands to polyNIPAAm, Protein A should couple well, as the lysine residues are apparently not important for the interaction with the antibodies, since their alkylation does not destroy the biological activity [13]. Protein A binds specifically to the Fc portion of IgG from several

species like human, rabbit, and mouse (figure 5.2). No interaction occurs with the Fab fragment, leaving this domain free for the binding of the antigen. Protein A consists of five regions, namely four highly homologous domains that bind to the Fc-region, whereas the fifth, C-terminal domain is normally bound to the cell wall [15]. However, apparently only two sites are implicated at any given moment in the interaction between Protein A and IgG [16].

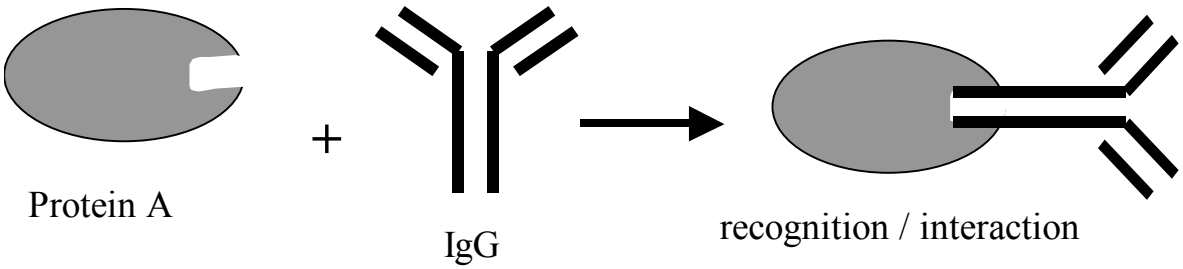


Figure 5.2. Affinity interaction between the Protein A and the Fc region of the IgG.

Despite the high binding capacity and selectivity of Protein A, this affinity ligand suffers from several drawbacks, such as complexity of its isolation and purification from microbial extracts, the presence of biological contaminants that are very difficult to eliminate, the high cost and the low stability to sanitation agents used for pyrogen removal [17]. Another point is that Protein A is considered as toxic (potentially cancerogenic) and its use in purification process can produce contaminations of the final product (bleeding of Protein A from the stationary phase). To circumvent these problems, synthetic molecules with the same properties as Protein A but without the inconveniences has been developed. An example of such a molecule is the Protein A mimetic (PAM) indicated in figure 5.3.

Figure 5.3. (A) Amino acid sequence of PAM as given by Fassina et al. for potential substitution of Protein A in antibody purification [17]. (B) PAM modified at the C-terminus for a specific coupling to a solid phase via the SH-group of the amino acid cysteine.

The potential applications of Protein A and its mimics for the isolation of IgG 4E11 antibodies using affinity precipitation were studied. Protein A – polyNIPAAm bioconjugates were constructed by direct coupling as described in chapter 1, figure 1.5. The AML containing PAM was designed by a two step coupling protocol, preparing first an avidin-polyNIPAAm (AML precursor) and then adding the biotinylated PAM as shown in figure 5.4.

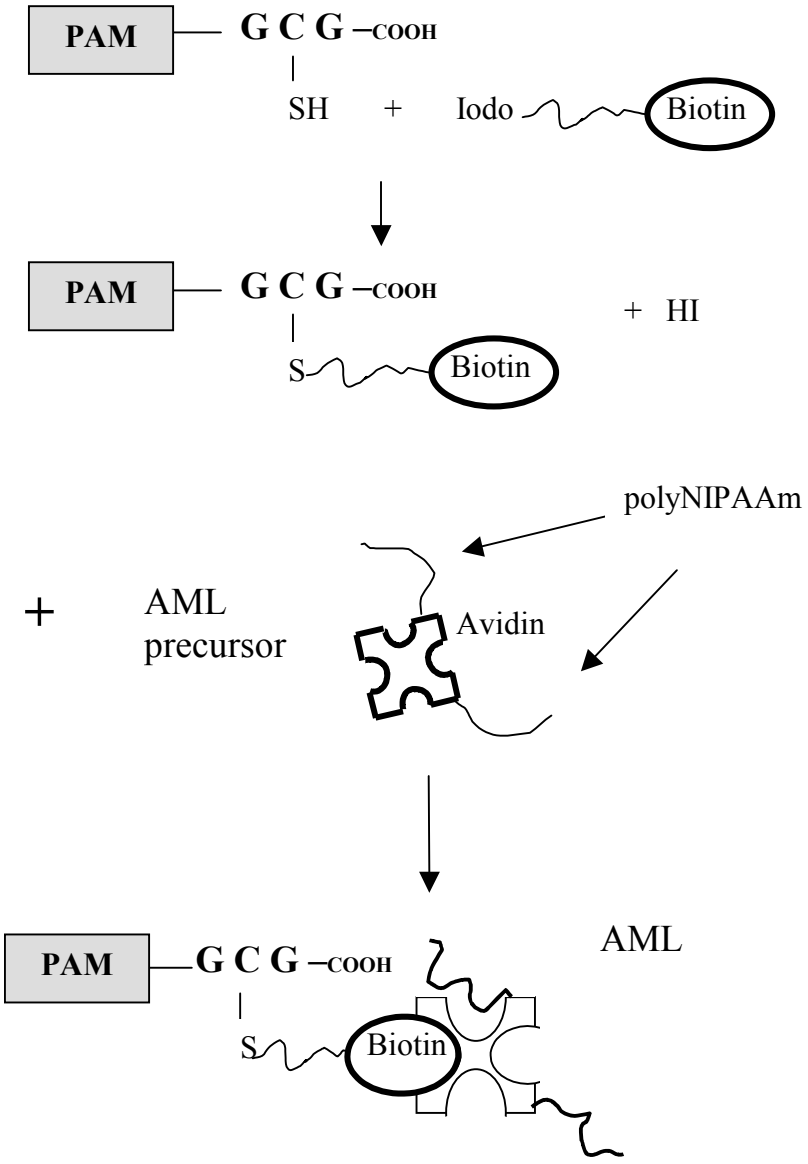


Figure 5.4. Design of the Protein A mimic (PAM)-containing AML. A previous biotinylation of the PAM was prepared using iodoacetyl biotin.

The FLAG peptide is a frequently used fusion tag, which was specifically designed to facilitate rapid purification of fusion proteins by affinity chromatography using the anti-FLAG antibody 4E11 as affinity ligand [18]. It has eight amino acids (sequence H_2N-**DYKDDDDK**-COOH), which can be placed at the N- or C- terminus of the target protein [19]. The strong hydrophilicity of the sequence KDDDDK has been shown to ensure strong antibody binding as it presents a highly exposed motif in the tertiary structure of the protein

[20, 21]. The last five amino acids (DDDDK) represent the minimal enterokinase specifity site, thus enabling the proteolytic removal of the tag [18].

The monoclonal antibody M1, also called IgG 4E11 (anti FLAG-tag, murine, monoclonal, IgG_{2B}-subtype) recognizes the free N-terminus of the FLAG-tag in a calcium dependent manner [21]. Dissociation of the complex can be performed by addition of chelating agents such EDTA (non-denaturing conditions) [22, 23]. This effect can be exploited for immunoaffinity purification of FLAG-tagged fusion proteins. In an inversion of the usual approach, the potential application of the FLAG-tag for the isolation of IgG 4E11 antibodies using the affinity precipitation approach with different AML of the FLAG-tag - polyNIPAAm bioconjugates was studied here.

5.2 Materials and Methods

5.2.1 Materials

Avidin (from hen egg white, affinity purified), biotin-labeled Protein A, biotin (USP grade), 4-hydroxyazobenzene-2-carboxylic acid (HABA), buffer salts, and organic solvents were obtained from Sigma-Aldrich. The highest available quality was used throughout. Purified IgG 4E11 (anti FLAG-tag antibody) was supplied by our cell culture group (Laboratory of Chemical Biotechnology, EPFL, Switzerland). N-α-Fmoc protected amino acids, preloaded resins, and the activator 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) were from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). N-Iodoacetyl-N-biotinylhexylene diamine (Iodoacetyl-Biotin) was from Uptima, Interchim (Montluçon Cedex, France). Unless indicated otherwise, all substances were used as obtained from the supplier. Water was purified with an Elix-3 water purification system (Millipore, USA).

5.2.2 Analytical Methods

All absorbance measurements were done in a Lambda 20 spectrophotometer (Perkin Elmer, Norwalk, CT). The avidin content of a given sample was estimated spectrophotometrically by UV absorption at 280 nm and by Green's HABA assay (see ref. [24, 25]). The remaining biotin binding sites in avidin and the amount of biotin present in solution (e.g. for biotinylated peptides) were also determined by the HABA assay. The peptide content of a given sample

was measured at 280 or 214 nm. The masses of the peptides were determined by electrospray ionization mass spectrometry with time-of-flight detection on a LCT mass spectrometer from Micromass (Manchester, UK). The amount of Protein A was quantified using the Micro BCA Protein Assay (Pierce), according to the manufacturer's instructions [26]. Samples were diluted in Working Reagent and prepared in test tubes. The absorbance was quantified in a spectrophotometer at 562 nm. Standard curves were generated using bovine serum albumin (BSA, Sigma-Aldrich). Standards and samples were measured in duplicate. Biacore (Biacore AB, Uppsala, Sweden) experiments were done with biotinylated Protein A on a Biacore streptavidin sensor chip according to the manufacturer's instructions [27]. IgG samples and myoglobin (negative control) were analysed to determine the affinity constant of the IgG binding.

Total antibody concentrations in purified solution were determined spectrophotometrically assuming that 1 mg/mL gives an OD_{280nm} of 1.551 (Dr. Caroline Vandevyver, personal communication). The IgG 4E11 concentration was also determined by a mouse IgG ELISA as follows. Microtiter plates (Nunc-Immuno™ plates, Maxisorp; Nunc International) were coated with 100 µL per well of a 2 µg/mL purified Rabbit anti-Mouse IgG (Code No Z0109, DAKO, Glostrup, Denmark) solution in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02 % NaN_3, pH 9.6) and incubated for 30 min at room temperature under agitation (600 rpm, Titramax 101 Shaker, Heidolph Instruments, Schwabach, Germany). The plates were washed 3 times with wash solution (150 mM NaCl in water containing an additional 0.1 % Tween-20) and then blocked with 200 µL per well of blocking buffer (1 % (w/v) Casein, 0.05 % (w/v) Tween-20 in PBS, where PBS is composed of 8.2 mmol Na_2HPO_4, 1.5 mmol KH_2PO_4, 137 mmol NaCl, and 2.7 mmol KCl in 1 L water, pH 7.4) for 15 min at 600 rpm. The plates were washed 3 times as described above. 100 µL from the IgG-containing samples (from the affinity precipitation / chromatography method) or 100 µL of a purified mouse IgG standard (Mouse anti-Human Kappa Light Chain, Cat. No AHI0801, BioSource Invitrogen, Carlsbad, California, USA) or 100 µL of the blank (i.e. only blocking buffer, no antigen) were added per well. Standards were measured in duplicates, starting at 200 ng/mL and diluted in a series of 8 two-fold dilutions in blocking buffer. IgG-containing samples (from the affinity precipitation / chromatography) were also diluted (8 two-fold dilutions) in blocking buffer. The plates were incubated for 30 min at room temperature and shaken at 600 rpm. The plates were washed 3 times as described above and 100 µL of a solution (a 5000x stock solution diluted 5-fold) of Rabbit anti-Mouse IgG HRP (Code No P0260, DAKO) in blocking buffer

were added per well and incubated for 30 min at room temperature (600 rpm). The plates were washed 6 times as described above. 100 µL per well of 1-Step ABTS (Pierce) ready-to-use substrate solution were added and the plate incubated for 15 to 60 min in the dark at room temperature and gently shaken at 450 rpm for development. The absorbance was measured at 405 nm using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A)

5.2.3 M1 Anti-FLAG antibody (4E11)

5.2.3.1 Antibody production

The M1 Anti-FLAG antibody was supplied by Dr. Caroline Vandevyver from the cell culture group of the Laboratory of Chemical Biotechnology, EPFL, Lausanne Switzerland. It was produced in the 4E11 hybridoma cell line (HB-9259, ATCC, US). The culture was made in suspension in Nunc cell factory devices (Nalge Nunc, Neerijse, BE). The propagation of the cells was done in CD hybridoma culture medium (Gibco BRL, Paisley, UK) in a 37 °C incubator with CO_2 set to 5 %. The produced antibodies were harvested every 3-4 days. Cells were removed by centrifugation at 2'500 *g* for 15 min. The supernatants were preliminary Protein A purified with a well established Protein A affinity chromatography protocol or were stored at –20 °C. The total protein concentration was determined at 280 nm assuming that after Protein A chromatography the antibody is almost pure (1 mg/mL gives an OD_{280nm} of 1.551, as mentioned before), and the IgG 4E11 concentration was determined by the mouse ELISA test.

5.2.3.2 Preliminary antibody purification from culture supernatants by Protein A affinity chromatography

The antibody purification from cell culture supernatans was done by Stéphane Canarelli from the biopurification group of the Laboratory of Chemical Biotechnology, EPFL, Lausanne Switzerland. It was performed on a FPLC system from Amersham Biosciences (Uppsala, Sweden), consisting of a 8 port valve, two P-500 syringe pumps, and a liquid chromatography controller LCC-500-plus. The samples were injected with a third P-500 syringe pump from the same supplier, due to the large injection volume of the sample. Detection was by a UV detector S-3702 (Soma Optics Ltd., Tokyo, Japan) with the wavelength set to 280 nm. A HR 16/10 empty column packed with rProtein A Sepharose® Fast Flow (stationary phase, [28]) was used (both from Amersham Biosciences). The conditioned culture medium (400 mL),

after cell removal, now was directly loaded into the affinity column at a flow rate of 1 mL/min. After that the column was washed with binding buffer (PBS 50 mM pH 7.4, 120 nM NaCl) until the monitored UV signal reached the initial baseline level. The elution of the antibody was performed with 0.1 M glycine-HCl pH 3 and the eluate was directly neutralized with 0.5 M Na_2HPO_4 pH 8 buffer. The washing and elution steps were always performed at a flow rate of 1 mL/min. The total protein of the eluate fractions was determined spectrophotometrically at 280 nm and the mouse IgG 4E11 concentration by the mouse ELISA test.

5.2.4 Synthesis of biotinylated PAM / FLAG-tag peptides

All peptides were synthesized using an AMS 422 peptide synthesizer from ABIMED (Langenfeld, Germany). The procedure of synthesis, cleavage and purification of the peptides was as described in chapter 3. However, the synthesizer was charged with preloaded Fmoc-Gly-wang resin (0.78 mmol/g) providing the C-terminus of the peptides (see figure 5.3 and table 5.1). In the case of the branched peptide (PAM) the synthesis protocol was slightly modified as a Fmoc-Lys(Fmoc)-OH was used instead of the Fmoc-Lys(Boc)-OH. A normal coupling was done for each amino acid on the linear sequence (KGGGGGC). For the branches, the coupling step was repeated as many times (2 or 4) as there were branches, without deprotection in between, to assure that all branches add the amino acid.

Table 5.1. FLAG peptides synthesized. The reactive group (sulfhydryl group on cysteine, C) on the FLAG peptides participating in the coupling to the biotin, which will be further attached to the AML-precursor (Avidin-polyNIPAAm) is underlined.

FLAG-1: H_2N-**DYKDDDDKG<u>C</u>G**-COOH	FLAG-2: H_2N-**DYKDDDDKGGGGG<u>C</u>G**-COOH

Peptides having a cysteine amino acid (sulfhydryl group) were biotinylated with Iodoacetyl-Biotin as shown in figure 5.4 (first step), and were again purified following the procedures described in chapter 3.

5.2.5 Affinity Chromatography

5.2.5.1 Immobilization of cysteine-bearing peptides on a SulfoLink Coupling Gel column

FLAG-1, FLAG-2 and PAM were coupled to the SulfoLink Coupling Gel Support (Pierce) according to the manufacturer's instructions [29]. Briefly, the sulfhydryl-containing peptide (1.9 mg (1.5 µmol) of FLAG-1, 4.0 mg (2.7 µmol) of FLAG-2, 6.7 mg (2.6 µmol) of PAM) dissolved in 2 mL of SulfoLink Coupling Buffer was loaded onto a 2 mL SulfoLink Coupling Gel column, mixed by rocking for 15 min at room temperature and incubated at room temperature for 30 min without mixing. The column was washed with 6 mL of SulfoLink Coupling Buffer. The absorbance at 280 nm (tyrosine (Y) absorbance), before and after coupling, was measured to determine the coupling efficiency. Non-specific binding sites on the gel were blocked by passing 2 mL of a 0.05 M cysteine solution in SulfoLink Coupling Buffer through the column, mixed by rocking for 15 min at room temperature and incubated for 30 min at room temperature without mixing. The column was washed with 4 x 4 mL of SulfoLink Wash Buffer and with 3 x 4 mL of a 0.05 % sodium azide (NaN_3) solution in water. The column was kept in 0.05 % degassed NaN_3 solution in water for further use.

5.2.5.2 Isolation of IgG by affinity chromatography (PAM / FLAG-tag)

Affinity chromatography was done for antibody purification according to the manufacturer's instructions [29] using binding, washing and elution conditions proposed in the literature [18, 19, 21, 23]. Briefly, the column with immobilized FLAG-tag/PAM was equilibrated with 6 mL of binding buffer (FLAG-1 and FLAG-2 used TBS pH 7.4 (50 mM Tris-HCl, 150 mM NaCl) with 10 mM $CaCl_2$, PAM used 0.2 M phosphate buffer pH 7.4). 1 mL of IgG-containing sample (1.12 mg IgG 4E11/mL in binding buffer) was loaded into the column, followed by 0.2 mL of binding buffer. For FLAG-tag experiments, the IgG-containing sample was supplemented with a 1 M $CaCl_2$ solution to reach a final molarity of 10 mM in Ca^{2+} before loading into the column. 1 mL of binding buffer were applied and incubated on the column at room temperature for 1 h. The column was washed with 16 mL of binding buffer. The bound compounds were eluted with 8 mL of elution buffer (0.1 M glycine-HCl pH 3) and 1 mL fractions were collected and immediately neutralized with 0.5 M Na_2HPO_4, pH 8 buffer. The column was regenerated with 16 mL PBS and stored at 4 ˚C in 0.05 % NaN_3 for further

use. The total protein was determined spectrophotometrically at 280 nm and the mouse IgG 4E11 concentration by the mouse ELISA test.

5.2.6 Affinity precipitation

5.2.6.1 Construction of the AML

The stimuli-responsive polymer coupled to Avidin was obtained as described in chapter 2. The first Protein A-AML, namely Protein A-AML 1, was prepared via the avidin/biotin-interaction as follows (table 5.2): 15 µL of a 2 mg/mL biotinylated Protein A solution (4 biotin per Protein A molecule as indicated by Sigma) in 0.2 M phosphate buffer (pH 8) was first incubated with 120 µL of a 10.6 mg/mL avidin-activated AML-precursor in water at a molar ratio avidin : biotin of 4 to 1. The solution was gently stirred overnight at 4 °C. The next morning, 200 µL of a 0.071 mg/µL non-activated polyNIPAAm solution in 0.2 M phosphate buffer (pH 8) were added, incubated and gently stirred for 1 h at 4 °C (total polyNIPAAm concentration: 4.3 % w/v). The AML was recovered by a thermocycling step (precipitation with 1/8 or 1/4 vol of a saturated $(NH_4)_2SO_4$ solution at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). The amount of saturated aqueous ammonium sulphate solution added was 40 µL and the final polyNIPAAm concentration 3.8 % w/v. The AML was purified by repeated thermocycling from fresh 0.2 M phosphate buffer, pH 7.4. The Protein A-content of the AML was determined by measuring the Protein A-content in all recovered supernatants and then substracted from the initial amount of Protein A. The number of remaining free binding sites on avidin were determined with the HABA test.

The construction of the others AML (e.g. PAM, FLAG-1 and FLAG-2) via the AML-precursor followed the same procedure as for the Protein A-AML 1 and the amounts of reagents used are also indicated in table 5.2.

Table 5.2. Conditions for AML synthesis (via the Avidin-activated AML-precursor)

	Protein A-AML 1	PAM AML	FLAG-1 AML	FLAG-2 AML
µmol biotinylated capture molecule	0.0046 [a]	0.1004	0.0224	0.0976
mg biotinylated capture molecule	0.050 [b]	0.293	0.036	0.180
in final vol µL [c]	15	250	50	250
µmol avidin (AML-precursor)	0.019	0.023	0.023	0.023
in final vol µL [c]	120	160	200	160
initial molar ratio biotin : avidin	1 : 4	4.4 : 1	1 : 1	4.2 : 1
initial molar ratio biotin : binding sites in avidin	1 : 10	1.7 : 1	1 : 2.5	1.7 : 1
mg non-activated polyNIPAAm	14.3	29.2	25.0	29.2
in final vol µL [c]	200	350	300	350
saturated $(NH_4)_2SO_4$ solution (µL)	40	190	138	190
Final total volume (µL)	375	950	688	950
% (w/v) polyNIPAAm	3.8	3.1	3.6	3.1

[a] µmol of biotin in the biotinylated Protein A. Knowing that there is an average of 4 biotin conjugated to the Protein A molecule, this value correspond to 0.0011 µmol of Protein A. [b] This value corresponds to 0.0011 mg of biotin and 0.0489 mg of Protein A. [c] 0.2 M phosphate buffer pH 8.

A second Protein A-AML, namely Protein A-AML 2, was prepared by direct coupling of polyNIPAAm to Protein A as follows: 0.3 mg of activated polyNIPAAm (30 mol equiv) were dissolved in 133 µL of dry DMF and added incrementally at 4 °C to a gently stirred solution of 0.2 mg Protein A in 40 µL of a 0.5 M phosphate buffer (pH 8.1). 627 µL of 0.2 M borate buffer (pH 8.7) were added and the solution was gently stirred overnight at 4 °C. The next morning, 50 mg of non-activated polyNIPAAm were added and gently mixed in (total polyNIPAAm concentration: 6 % w/v). The AML was recovered by a thermocycling step (precipitation with ¼ vol of the saturated $(NH_4)_2SO_4$ solution at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). The amount of saturated aqueous ammonium sulphate solution added was 200 µL and the final polyNIPAAm concentration 5 % w/v. The non-coupled activated polyNIPAAm was blocked by redissolution of the AML in fresh 0.2 M borate buffer, pH 8.7

at 4 °C containing 0.5 M ethanolamine followed by a thermocycling step. The AML was then redissolved in fresh 0.15 M NaOH at 4 °C (hydrolization of NHS) followed by a thermocycling step. The AML was purified by repeated thermocycling from fresh 0.2 M borate buffer, pH 8.7. The Protein A-content of the AML was determined by measuring the Protein A-content in all recovered supernatants (unbound Protein A) and then substracted from the initial amount of Protein A.

5.2.6.2 Isolation of IgG by affinity precipitation (Protein A / PAM / FLAG-tag)

The Protein A-AML 1 was used for the affinity precipitation of IgG as follows (table 5.3): 150 µL of Protein A-AML 1 (0.039 µg Protein A/µL, 48 µg polyNIPAAm /µL, in binding buffer (0.2 M phosphate buffer pH 7.4)) solution, were mixed with 100 µL of IgG solution (430 µg IgG 4E11/mL in neutralized elution buffer after Protein A chromatography (0.1 M glycine-HCl neutralized with 0.5 M Na_2HPO_4, pH 8 buffer). 50 µL of binding buffer were added before incubation at 4 °C overnight under gently mixing. 100 µL of a non-activated polyNIPAAm solution (71 µg/µL, in 0.2 M phosphate buffer pH 7.4) were added and gently mixed at 4 °C. The IgG-AML complexes were recovered by a thermocycling step (precipitation with 40 µL of a saturated $(NH_4)_2SO_4$ solution at 37 °C / centrifugation at 37 °C, 10'000 *g*, 15 min). The final polyNIPAAm concentration was 3.2 % w/v. The IgG-AML complexes were washed by redissolution of the complex (pellet) in fresh binding buffer (400 µL) at 4 °C followed with a thermocycling step in the presence of 40 µL of saturated $(NH_4)_2SO_4$) solution. For recovery of the IgG, an elution step was done by redissolving the IgG-AML complexes in 250 µL of fresh elution buffer (0.1 M glycine-HCl, pH 3) followed by a thermocycling step (removal of the AML), recovering the supernatant and immediatly adding 0.5 M Na_2HPO_4, pH 8 buffer solution for neutralization. The total protein concentration was determined spectrophotometrically at 280 nm and the mouse IgG 4E11 concentration by the mouse IgG ELISA test.

The same procedure was followed with the other AML (PAM, FLAG-1, FLAG-2 and Protein A-AML 2) investigated. The amounts of reagents, the binding, washing and elution buffers used are indicated in table 5.3.

Protein A-AML 1 was also used for a binding / elution study when increasing amounts (0, 4, 8, 20, 40 and 60 pmol) of a 0.9 pmol Protein A/µL solution in 0.2 M phosphate buffer pH 7.4 were incubated 2 h at 4 °C with 40 pmol of a 2.9 pmol IgG 4E11/µL solution in neutralized

elution buffer after Protein A chromatography (0.1 M glycine-HCl neutralized with 0.5 M Na_2HPO_4, pH 8 buffer). The same affinity precipitation procedure as described before was followed. For these experiments, the final concentration of non-activated polyNIPAAm was 3.1 % (w/v). 20 µL of a saturated $(NH_4)_2SO_4$ solution were added during all precipitation steps and the final total volume was always 200 µL. The IgG 4E11 concentration was determined by the mouse IgG ELISA test.

Table 5.3. Amounts and conditions used for the affinity precipitation.

	Protein A-AML 1	PAM AML	FLAG-1 AML	FLAG-1 AML	FLAG-2 AML	Protein A-AML 2
µmol biotinylated capture molecule	1.3×10^{-4} [a]	0.0068	8.9×10^{-4}	8.9×10^{-4}	0.0068	6.6×10^{-4} [g]
mg biotinylated capture molecule	0.0059 [b]	0.0197	0.0014	0.0014	0.0124	0.0297 [h]
mg polyNIPAAm	7	3.5	1.0	1.0	3.5	16
in final vol µL binding buffer	150 [c]	60 [c]	20 [d]	20 [d]	60 [d]	80 [c]
µg IgG 4E11/mL	430	140	10	1.46	140	430
µL IgG 4E11 solution [e]	100	400	400 [f]	230 [f]	400 [f]	70
µmol IgG 4E11	2.9×10^{-4}	3.7×10^{-4}	2.7×10^{-5}	2.2×10^{-6}	3.7×10^{-4}	2.0×10^{-4}
initial molar ratio capture molecule : IgG	1 : 2.2	17 : 1	33 : 1	400 : 1	17 : 1	3.3 : 1
Extra µL binding buffer	50 [c]	0 [c]	50 [d]	240 [d]	250 [d]	150 [c]
mg non-activated polyNIPAAm	7	30	17	17	45	0
in final vol µL binding buffer	100 [c]	300 [c]	200 [d]	200 [d]	450 [d]	0 [c]
µL of saturated $(NH_4)_2SO_4$ sol.	40	190	80	80	290	40
Final total volume (µL)	375	950	750	770	1450	340
% (w/v) polyNIPAAm	3.2	3.5	2.4	2.3	3.3	4.7
washing buffer (µL) [i]	400	600	500	500	700	250
µL of saturated $(NH_4)_2SO_4$ sol.	40	150	60	60	175	40
% (w/v) polyNIPAAm	3.2	4.5	3.2	3.2	5.5	5.5
elution buffer (µL) [j]	250	600	500	500	700	250
µL of saturated $(NH_4)_2SO_4$ sol.	25	150	60	60	175	40
% (w/v) polyNIPAAm	5.1	4.5	3.2	3.2	5.5	5.5

[a] µmol of Protein A present in the AML. [b] mg of Protein A present in the AML. [c] 0.2 M phosphate buffer pH 7.4. [d] TBS pH 7.4 with 10 mM $CaCl_2$. [e] Protein A purified IgG, in neutralized elution buffer after Protein A chromatography. [f] with 10 mM $CaCl_2$. [g] µmol of Protein A present in the AML when directly coupled to the polyNIPAAm. [h] mg of Protein A present in the AML when directly coupled to the polyNIPAAm. [i] Use the binding buffer. [j] 0.1 M glycine-HCl buffer pH 3.

5.3 Results and Discussion

5.3.1 Affinity chromatography

Following the protocol described above for the coupling of FLAG-1, FLAG-2 and PAM to the SulfoLink gel, a coupling efficiency of respectively 70 %, 88.5 % and 85 % was obtained as estimated by the OD_{280nm}. The affinity chromatography protocol was done with an initial molar ratio peptides to IgG of around 300 to 1. In these experiments, it was used the same elution buffer (0.1 M glycine-HCl, pH 3) as it is already applied in current Protein A affinity chromatography protocols [30, 31]. The ELISA analysis of the eluted samples indicated that only 0.1 to 1.8 % of the IgG 4E11 was retained by the column giving between 10^{-4} and 10^{-5} mol IgG / mol capture molecule (FLAG-tag or PAM) under these circumstances. These low yields weren't expected and could be related to the denaturation (conformational changes) of the IgG after the elution at pH 3 [32], even if the neutralization was done immediately (Sigma protocols using anti-FLAG tag antibodies propose to not leave these antibodies for longer than 20 min in glycine-HCl [33]). IgG 4E11 antibodies are very sensitive to low pH [32] and if their structure is affected, the ELISA test will be necessarily affected [34]. For comparison, rProtein A Sepharose® Fast, used as stationary phase for Protein A chromatography, has a binding capacity of 8-20 mg mouse IgG/mL medium, with approximately 6 mg Protein A/mL medium [28, 31]. These values given by the manufacturer indicate a binding ratio of 0.4-1 mol IgG / mol Protein A. In my experiments, the column capacity by column overloading with IgG (target antibody in excess) wasn't measured.

5.3.2 Affinity Precipitation

Three batches of Avidin-activated AML-precursor were produced. The avidin coupling efficiency to the polyNIPAAm was found between 61 % to 85 % when using a molar ratio polyNIPAAm to avidin of 100 : 1. The HABA test indicated that 2-3 binding sites (a value of 2.5 was used for calcutations) on avidin were still free for linking the biotinylated capture molecules (Protein A, PAM, FLAG-1 and FLAG-2).

5.3.2.1 Use of Protein A as affinity ligand

In case of Protein A, two routes were investigated for the preparation of the AML. In the first case (Protein A-AML-1) a polyNIPAAm bearing avidin was prepared and linked to a

biotinylated Protein A according to the standard protocol [35]. Alternatively (Protein A-AML-2), the Protein A was linked directly to the carboxylic acid activated polyNIPAAm. In the first case a molar ratio (binding sites) between PolyNIPAAm-Avidin and Protein A-biotin of 17 to 1 was chosen. Under these circumstances 23.8 % of the biotinylated Protein A molecules were integrated into the AML as evidenced by the BCA assay. In the second case the molar ratio between COOH-polyNIPAAm and Protein A during covalent coupling was 30 : 1. Coupling efficiency was 74.1 % under these circumstances (BCA test measurement). The affinity of the Protein A ligand was determined by Biacore experiments and a dissociation constant of 2.8×10^{-7} M ($Ka = 3.5 \times 10^{6}$ M^{-1}) was found for the target antibody. This is in the general order of Protein A-antibody binding constants and almost one order of magnitude better than the value published by Chen and Hoffman (3×10^{-6} M) [36].

Figure 5.5 summarizes a binding study showing the respective amounts of IgG captured from solution by a given amount (in pmol) of Protein A-AML-1. The concentration of the target antibody was maintained at 40 pmol in these experiments. At low AML concentration, (target antibody in excess) approximately one molecule of antibody is capture per molecule of AML. If the AML concentration is increased (data not shown in figure 5.5) to be in excess, the maximum amount of antibody binding is defined by the affinity constant of the complex.

Comparing both Protein A-AML used, table 5.3 and table 5.4, Protein A-AML 1 showed a higher binding ratio (up to 1.03 moles of IgG bound/moles of Protein A) than Protein A-AML 2 (only 0.15 moles of IgG bound/moles of Protein A). In my experiments, the AML capacity by overloading with IgG (target antibody in excess) wasn't measured. I presume that this is due to an interference of the polyNIPAAm (direct coupling) with the antibody-binding site of Protein A, as it was also described by Chen and Hoffman experiments with a polyNIPAAm-Protein A conjugate [36]. Chen and Hoffman obtained a binding ratio of 0.25 moles of IgG bound/mole of Protein A.

In my case, if the binding ratio (Protein A / IgG) is increased (excess of AML), a higher IgG recovery from target molecules in solution was observed. For instance for a molar ratio of 1 : 1 Protein A-AML 1 binds 30,0 % of the target molecules in solution and for a molar ratio (Protein A to IgG) of 1.5 : 1 Protein A-AML 1 binds 35,0 %. In the case of Protein A-AML 2 for a molar ratio of 3.3 : 1, 49.5 % of the target molecules are bound.

When the incubation time during binding was decreased from overnight incubation to 2h (results in fig. 5.5), a lower binding ratio was obtained going from 1 to 0.5 for an initial molar ratio Protein A to IgG of 1 : 2. The expected binding ratio found in the literature is of 2 mol IgG per mol Protein A [16].

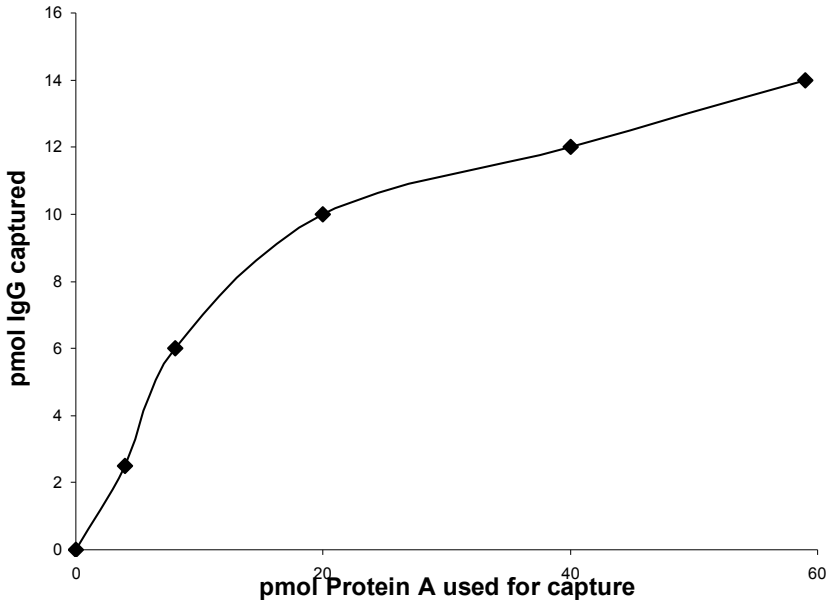


Figure 5.5. Capture of IgG 4E11 with increasing amounts of Protein A-AML-1. The target antibody was present in all samples at a total concentration of 40 pmol.

5.3.2.2 Use of PAM, FLAG-1 and FLAG-2 as affinity ligands

The biotinylation yield of the different peptides (PAM, FLAG-1, FLAG-2) was around 27 %. The iodoacetyl-biotin showed solubility problems during the biotinylation reaction and this could be the reason why the yield was not higher. However, enough material was obtained to proceed with the AML construction. The next step was the addition of the biotinylated peptide to the AML-precursor. In the case of FLAG-1, where the initial molar ratio biotin / free binding site in avidin was smaller than 1, it was possible to integrate 100 % of the biotinylated peptides into the AML and it was estimated one capture molecule per AML. It was also found that some binding sites in the avidin were still free as it was to be expected when working with this molar ratio. In the case of the PAM AML and the FLAG-2 AML, where the initial molar ratio biotin / free binding site in avidin was higher than 1, a saturation of the remaining binding site in avidin (after polyNIPAAM coupling) was obtained when 58

% of biotinylated molecules added were attached, which gave an average of 2.5 capture molecules per AML.

The constructed AML (PAM AML, FLAG-1 AML and FLAG-2 AML) were tested in affinity precipitation experiments and they were compared with previous results obtained with both of the Protein A-AML . The results are summarized in table 5.4.

Table 5.4. Results obtained with the affinity precipitation. Related to table 5.3

	Protein A-AML 1	PAM AML	FLAG-1 AML	FLAG-1 AML	FLAG-2 AML	Protein A-AML 2
µmol biotinylated capture molecule	1.3×10^{-4} [a]	0.0068	8.9×10^{-4}	8.9×10^{-4}	0.0068	6.6×10^{-4} [b]
µmol initial IgG 4E11	2.9×10^{-4}	3.7×10^{-4}	2.7×10^{-5}	2.2×10^{-6}	3.7×10^{-4}	2.0×10^{-4}
initial molar ratio capture molecule : IgG	1 : 2.2	17 : 1	33 : 1	400 : 1	17 : 1	3.3 : 1
mol IgG recovered / mol capture molecule	1.03	5.4×10^{-5}	2.5×10^{-3}	9.9×10^{-6}	5.4×10^{-5}	0.15
IgG Yield recovery % [c]	46.2	0.1	8.4	0.4	0.1	49.5
IgG Yield recovery % [d]	-	-	0.5	2.0×10^{-3}	1.1×10^{-2}	-

[a] µmol of Protein A present in the AML. [b] µmol of Protein A present in the AML when directly coupled to the polyNIPAAm. [c] (IgG recovered / IgG initially added) x 100. [d] Only done for FLAG-tag AML, (IgG recovered / theoretical maximum IgG recovered) x 100, using a theoretical maximum binding ratio of 2 : 1 (FLAG-tag / IgG 4E11) as indicated in ref. [23].

When these AML were used for the isolation of the target molecule in affinity precipitation experiments, their performance were not satisfactory. Even if an excess of capture molecules were used over target molecules, the recovery yields after elution were only between 0.1 % and 8.4 % of the initial IgG amount, which corresponds to 10^{-6} to 10^{-3} mol IgG purified per capture molecule in the AML. Prickett et al. [23] obtained a yield between 15-40 % of the theoretical maximum assuming a 2 : 1 antigen (FLAG) to antibody (4E11) binding ratio, while in my experiments, when using the FLAG-tag AML, the yields were only between 0.002-0.5 %. For comparison, the affinity chromatography using the same capture molecules (FLAG-tag or PAM) also gave unsatisfactory results as only 0.1 to 1.8 % of the IgG 4E11

was retained by the column giving between 10^{-4} and 10^{-5} mol IgG / mol capture molecule (FLAG-tag or PAM).

The sequence of the peptides shouldn't be the reason of the problem as the MS analysis of all synthesized peptides indicated that the correct peptides were synthesized. As the experimental conditions were adapted from those found in the literature, this shouldn't be either the main reason of these unsatisfactory results.

Such low yields could be explained assuming that the antibody was denatured, probably during the acidic pH elutions done with 0.1 M glycine-HCl (pH 3): first "contact" during the purification of the antibody from culture supernatant (Protein A affinity chromatography) and then the "contact" during the affinity precipitation/chromatography tests. When the IgG concentration in eluate samples was measured using the two quantification methods, it was observed that the ELISA results were up to 99 % lower than those obtained with the OD_{280nm} even though we assumed the protein fraction of these samples contains mostly IgG. This can be caused by the denaturation of the IgG 4E11 affecting the interaction between the antibody and the capture molecule (IgG doesn't recognize anymore the binding site on FLAG-tag / PAM), can a similar problem may occur in the interaction between the purified antibody (mouse IgG 4E11) and those antibodies from the ELISA test (polyclonal rabbit anti-mouse IgG). The time required for the elution step in affinity precipitation was at least 20 min (optimization wasn't done), which was not convenient for the stability of the antibody as it was indicated in ref. [33], but the affinity chromatography (with a shorter time during elution step) was also not able to give a better purification. This means for me that the problem comes mainly from the initial IgG sample which seems to be already "damaged" (stressed / partly denatured).

Apparently, only experiments done with the IgG sample at the concentration of 430 µg/mL were fine (for both Protein A-AML). All others IgG samples gave unsatisfactory results. For one reason, the antibodies from this batch weren't denatured (no conformational changes) during Protein A chromatography elution neither on Protein A affinity precipitation elution steps.

Results shown in table 5.4 indicate that affinity binding sites on IgG for Protein A don't seem to be really affected by the acidic pH elution conditions. However, neither PAM affinity

chromatography nor PAM affinity precipitation results were able to confirm that PAM can mimic Protein A. Fassina's experiments [17] with a PAM column overloaded with rabbit IgG obtained a binding capacity up to 1.15 mg of rabbit IgG per mg of PAM (1.6×10^{-2} mol of IgG per mol of PAM).

Recently, Costioli [37] investigated the affinity precipitation for the purification of the IgG 4E11 using a different approach for the construction of the FLAG-tag AML (a direct telomerization approach using a modified FLAG-peptide as telogen). In this case as well, the majority of the target antibody remained in solution after the affinity precipitation process. Practically no target molecules were found in the released supernatant (the elution was also done with 0.1 M glycine-HCl pH 3 followed by a neutralization step). The IgG concentration was determined by UV absorption at 280 nm. Costioli tried to improve the apparent lack of interaction between the AML and the antibody by modifying some parameters of the capturing step, but nevertheless the interaction seems to be unattainable.

5.4 Conclusion

Five AML were tested in affinity precipitation for IgG purification. The best results were obtained with the Protein A-AML, with nearly the expected ratio as found in the literature. Two approaches were used for the construction of the Protein A-AML and it was observed that the direct coupling of polyNIPAAm reduced the binding ratio compared to the AML obtained by an avidin-biotin interaction. The major problem is the control of the coupling of polyNIPAAm to the affinity tag to avoid the blocking of the binding sites responsible for the affinity interaction. The Avidin-Biotin interaction is a possibility to bypass this problem but will result in a bigger and also more costly AML. A peptidic Protein A mimic was also tested in affinity precipitation but the results weren't satisfactory, although not worse than those for affinity chromatography using this ligand. FLAG-tag peptides were tested in affinity precipitation / chromatography for IgG 4E11 purification, but again no satisfactory antibodies recovery was obtained. It seems that stressed (partly denatured) antibodies were used, affecting the interaction between capture molecules and IgG, but also giving an underestimation of the ELISA results. However, the first results with affinity precipitation of antibodies are promising but need to be further optimization in regard to the elution conditions and the quantification methods.

5.5 References

1. Bodey, B.; Kaiser, H.E.; Goldfarb, R.H. Immunophenotypically varied cell subpopulations in primary and metastatic human melanomas. Monoclonal antibodies for diagnosis, detection of neoplastic progression and receptor directed immunotherapy. Anticancer Res., **1996**, 16, 517-532.
2. Longo, D.L. Immunotherapy for non-Hodkin's lymphoma. Curr. Opin. Oncol., **1996**, 8, 353-359.
3. Fahrner, R.L.; Blank, G.S.; Zapata, G.A. Expanded bed protein A affinity chromatography of a recombinant humanized monoclonal antibody: process development, operation, and comparison with a packed bed method. J. Biotechnol., **1999**, 75, 273-280.
4. Freitag, R.; Horvath, C. Chromatography in the downstream processing of biotechnological products. Adv. Biochem. Bioeng., **1995**, 53, 17-59.
5. Thömmes, J.; Bader, A.; Halfar, M.; Karau, A.; Kula, M.R. Isolation of monoclonal antibodies from cell containing hybridoma broth using a protein A coated adsorbent in expanded beds. J. Chrom. A., **1996**, 752, 111-122.
6. Pier, G.B.; Lyczak, J.B.; Wetzler, L.M., Immunology, infection and immunity, in Immunology and cell biology. **2004**, 82, Blackwell Publishing: Oxford. 651.
7. Junqueira, L.C.; Carneiro, J.; Kelley, R.O., *Basic Histology*. 9th Ed ed, ed. McGraw-Hill. **2003**, Norwalk, Conn.: Appleton and Lange.
8. Lehninger, A.; Nelson, D.; Cox, M., *Principes de biochimie*. 2nd Ed ed. Medicine-Science, ed. Flammarion. **1993**.
9. Stryer, L., in Biochemistry, 4th Ed. **1995**, Freeman: San Francisco.
10. Subramanian, A.; Velander, W.H. Effect of antibody orientation on immunosorbent performance. J. Mol. Recogn., **1996**, 9, 528-535.
11. Janeway, C.A., Jr.; Travers, P.; Walport, M.; Shlomchik, M.J., *Immunobiology: the immune system in health and disease*. 5th Ed ed. **2001**, New York: Garland Publishing.
12. *Antibody structure*. Available from: http://www.biology.arizona.edu/immunology/tutorials/antibody/structure.html.
13. Surolia, A.; Pain, D.; Khan, M.I. Protein A: nature's universal anti-antibody. Trends Biochem. Sci., **1982**, 7, 74-76.
14. Langone, J.J., Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by Streptococci and Pneumonococci, in Advances in Immunology, F.J. Dixon and H.G. Kunkel, Editors. **1982**, 32, Academic Press: New York. 157-252.
15. Mota, G.; Ghetie, V.; Sjöquist, J. Characterization of the soluble complex formed by reacting rabbit IgG with protein A of S. aureus. Immunochemistry, **1978**, 15, 639-642.
16. Sjöquist, J.; Meloun, B.; Hjelm, H. Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin. Eur. J. Biochem., **1972**, 29, 572-578.
17. Fassina, G.; Verdoliva, A.; Odierna, M.R.; Ruvo, M.; Cassini, G. Protein A mimetic peptide ligand for affinity purification of antibodies. J. Mol. Recogn., **1996**, 9, 564-569.
18. Einhauer, A.; Jungbauer, A. Affinity of the monoclonal antibody M1 directed against the FLAG peptide. J. Chrom. A., **2001**, 921, 25-30.
19. Hopp, T.P.; Prickett, K.; Price, V.L.; Libby, R.T.; March, C.J.; Cerretti, D.P.; Urdal, D.L.; Conlon, P.J. A short polypeptide marker sequence useful for recombinant protein identification and purification. Biotechnology, **1988**, 6, 1204-1210.
20. Hopp, T.P.; Woods, K.R. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA, **1981**, 78, 3824-3828.

21. Hopp, T.P.; Gallis, B.; Prickett, K. Metal-binding properties of a calcium-dependent monoclonal antibody. Mol. Immunol., **1996**, 33, 601-608.
22. Einhauer, A.; Jungbauer, A. The FLAG peptide, a versatile fusion tag for the purification of recombinant proteins. J. Biochem. Biophys. Methods, **2001**, 49, 455-465.
23. Prickett, K.S.; Amberg, D.C.; Hopp, T.P. Researc report: A calcium-dependent antibody for identification and purification of recombinant proteins. BioTechniques, **1989**, 7, 580-589.
24. Green, N.M. Specrophotometric determination of avidin and biotin. Methods Enzymol., **1970**, 18A, 418-424.
25. Hilbrig, F.; Stocker, G.; Schläppi, J.-M.; Kocher, H.; Freitag, R. Utilisation of group specific ligands in the downstream processing of proteins by affinity precipitation. Food Bioprod. Process., **2006**, 84 (C1), 1-9.
26. *Micro BCA Protein Assay, Pierce*. Available from: http://www.piercenet.com/files/0412dh5.pdf.
27. *Biacore*. Available from: http://www.biacore.com.
28. *rProtein A Sepharose Fast Flow, Amersham Pharmacia*. Available from: http://www1.gelifesciences.com/aptrix/upp00919.nsf/Content/BFD2D679531AF76AC1256EB40044AC95/$file/71500009AC.pdf.
29. *Sulfolink, Pierce*. Available from: http://www.piercenet.com/files/1980dh5.pdf.
30. *Protein A-Agarose, Sigma product information*. Available from: http://www.sigmaaldrich.com/sigma/product%20information%20sheet/pa1pis.pdf.
31. *Antibody Purification Handbook*. **2002**: Amersham Biosciences.
32. Vandevyver, C.; Freitag, R., Purification of antibodies by chromatographic methods, in Biopharmaceutical Antibodies: Production, Purification and Safety, S. Subramanian, Editor. **2003**, 1, Kluwer Academic/Plenum Publishers.
33. *Anti-FLAG M1, Sigma product information*. Available from: http://www.sigmaaldrich.com/sigma/datasheet/a4596dat.pdf.
34. *Mouse-IgG ELISA, Roche*. Available from: https://www.roche-applied-science.com/pack-insert/1333151a.pdf.
35. Freitag, R.; Hilbrig, F., Use of the avidin (imino)biotin system as general approach to affinity precipitation, in Avidin-Biotin Interactions, Methods and Applications, R.J. McMahon, Editor. **in press**, Methods in Molecular Biology, 418, The Humana Press Inc.: New Jersey.
36. Chen, J.P.; Hoffman, A.S. Polymer-protein conjugates. II. Affinity precipitation separation of human immunogammaglobulin by a poly(N-isopropylacrylamide)-protein A conjugate. Biomaterials, **1990**, 11, 631-634.
37. Costioli, M. Bioconjugates for bioseparation by affinity precipitation, **2004**, PhD Thesis EPFL, Lausanne

6. Final conclusions and perspectives

6.1 Final conclusions

Small changes in temperature induce the precipitation / resolublisation of polyNIPAAm (AML) as it is observed in CST measurements. Different AML were constructed using a "direct" coupling of polyNIPAAm to the capture molecule and a "indirect"coupling via the avidin-biotin interaction using an avidin-activated polyNIPAAm and a biotinylated capture molecule. This showed the polyvalent capacity of this bioseparation method. Preliminary affinity precipitation experiments have shown promising results. Target molecules could be selectively captured and purified from crude or partially purified samples by this method. Compared to standard technique (affinity chromatography, extraction via magnetic beads, selection by immunotubes), similar or even better purification yields were obtained and the quality of the purified molecules after dissociation from the AML were typically good. The determined affinity constants were nearly the expected values as found in the literature.

The affinity precipitation method takes advantages from working in homogeneous solution. Another advantage is the possibility to reduce the number of purification steps needed (compared to established affinity methods) by introducing the affinity precipitation at the beginning of the purification process. The method is easier to handle and to scale up. No dedicated equipment is necessary, only a water bath for heating / cooling and a standard laboratory centrifuge. It was possible to do the affinity precipitation in a sterile RNase-free environment as putative problems with RNases could be prevented by a suitable treatment of the AML-preparation. The resolublisation of the pellet after centrifugation was easier after the addition of some cellulose fiber before the precipitation step.

Even if the AML have compared low cost, the possibility for recycling was considered. When harsh conditions were necessary to disrupt the interaction between capture and target molecules, the integrity of capture molecules was affected (no recycling possible). A shorter incubation time at the elution conditions or comparatively mild elution conditions should be used instead.

6.2 Perspectives

The obtained results during this thesis are good enough as preliminary ones but of course they need additional improvements to reach better recovery yields. For that some points should be optimized like:

- The time necessary for the thermocycling step (precipitation and centrifugation), which needs to be reduced to obtain a faster method and also reduce the incubation times in case of harsh conditions.

- The number of polyNIPAAm molecules attached per AML should be determined.

- The optimum coupling ratio between polyNIPAAm and capture molecule should be found, to avoid the blocking by the polymer of important binding sites needed for the recognition of the target molecule. This will let to cost reductions for the construction of the AML.

- To scale up the process for higher samples volumes going from the mL to the liter scale. For this reason, it will be also interesting to use and test the filtration instead of the centrifugation for the separation of the solid phase.

Chemical Engineer EPFL

specialised in
Biotechnology

STOCKER MAJD Gisela

Av. du Silo 12
1020 RENENS
Office 021 693 61 08
Home 021 634 52 48
Mobile 076 548 50 43
gisela.stocker@epfl.ch

HANDS-ON EXPERIENCE TECHNOLOGY

- **Proteins / peptides**

  Solid phase peptide synthesis (Fmoc-chemistry): linear / branched peptides and their cleavage
  Phage Display: analysis of a scFv antibodies library against a peptide
  Quantification (spectrophotometry, BCA, NanoOrange, ELISA, Western-Blot)
  Analysis and characterization (SDS-PAGE, FPLC, HPLC, Biacore)
  Extraction and purification (precipitation, chromatography (affinity / size exclusion / reverse phase), dialysis)

- **Molecular biology**

  Extraction and amplification (PCR, RT-PCR)
  Quantification (UV-Vis, Picogreen, Luciferase/Luciferin) and analysis of DNA and poly(A) mRNA
  Analysis of phages DNA genome (Phage Display)
  Procedures were performed in a Dnase/Rnase free, sterile environment

- **Biotechnology**

  Experience in suspension cell culture (Jurkat)
  Experience in bacterial culture (*E. coli*) and with bacteriophages
  FACS analysis
  Procedures were performed in a sterile environment, laminar flow

- **Bioseparation methods**

  Purification of biomolecules using the affinity precipitation with thermoreponsive bioconjugates, the affinity chromatography, the magnetic beads and the immunotubes

KEY RESULTS

- **Proof of feasibility of a new method for bioseparation** (affinity precipitation) using bioconjugates constructed with polyNIPAAm (thermoresponsive polymer) and research of applications for purification / selection of biomolecules
- **Bioconjugates construction** (= polyNIPAAm + capture molecule) and application:

Capture molecule	Molecule to purify / select
Protein A	IgG 4E11 (Fc fragment)
Flag-tag linear et branched	IgG 4E11
Haptoglobin	Hemoglobin
Avidin-biotin-oligo (dT)	Poly(A) mRNA
Avidin-biotin-MUC1	scFv antibodies (Phage Display)

- Scientific publications:
  Stocker, G.; Vandevyver, C.; Hilbrig, F.; Freitag, R. Purification of RT-PCR competent poly(A) mRNA from crude cell lysate by affinity precipitation. Biotechnol. Prog., 2006, 22**,** 1621-1629.
  Hilbrig, F.; Stocker, G.; Schläppi, J.-M.; Kocher, H.; Freitag, R. Utilization of group specific ligands in the downstream processing of proteins by affinity precipitation. *Food Bioprod. Process.*, 2006, 84, 28-36.
  Stocker, G.; Dumoulin, D.;Vandevyver, C.; Hilbrig, F.; Freitag, R. Screening of a synthetic human antibody phage display library against the MUC-1 surface antigen using smart bioconjugates (Biotechnol. Prog., submitted)

WORK EXPERIENCE

2001-2006	Research engineer and PhD thesis at the Laboratory of chemical biotechnology, EPFL, Suisse
1995-2001	Different jobs during studies

OTHERS SKILLS

- Evaluated new method applied to biological purification process
- Defined validation protocols
- Collected documentation from various sources
- Written reports, process documentation, and scientific papers
- Taught newly introduced methods to colleagues and supported existing techniques
- Organized and managed technical development with master students
- Managed the running of the chemicals and biotechnology laboratories
- Contacted suppliers and purchased products / equipments

COMPUTER LITERACY

Operating systems: Microsoft Windows and Mac OS X
Softwares: Microsoft Office, FrameMaker, Micrografx Designer, Photoshop, Mathcad, AspenPlus
Internet: search engine and analysis tool for molecular biology

EDUCATION AND DIPLOMAS

June 2007	**PhD thesis** at the Laboratory of chemical biotechnology, EPFL, getting of the rank of **Docteur Es Sciences**
	PhD subject : « The affinity precipitation for the isolation of biomolecules»
March 2007	Certificate of lesson: Introduction to BPF and Hygiene. General knowledge on BPF and hygiene as well as pharmaceutical environment. Kelly Services (Nyon, Swiss)
2001	**Chemical engineer, EPFL**
	Options followed:
	Biotechnology engineering
	Industrial applications of biotechnology
	Master diploma subject « Study of the enzymatic activity of proteins conjugated to a thermoresponsive polymer (PNIPAAm) during cycles of precipitation / solubilisation at different temperatures »
1995	Special Mathematics Course (CMS) at EPFL
1993	French language course at the Ecole Bénédict and at the Université de Fribourg
1992	High school diploma in sciences (Panama)

LANGUAGES

(6 = excellent, 1 = notions/basic knowledge)

	Spanish (native language)	French	English	German	Swiss-German	Italian
Read	6	6	5	2	2	1
Write	6	5	4	2		
Talk	6	5	4	2	2	1

EXTRACURRICULAR ACTIVITIES

2004-2007	Voluntary first-aid worker at the EPFL
2000-2001	Fireman volunteer at Renens VD
1991-1992	Committee of the end of study (various activities to harvest funds)
1989	Committee of writing of a newspaper for the students (high school)

INTERESTS AND ACTIVITIES

Sports: Tennis, bicycle, hiking
Traveling: Meet others cultures (people, languages, food, landscape)
Others: Photography, creative activities like do-it-yourselfery and handicrafts

PERSONAL INFORMATION

Date of Birth: 23rd July 1975 (31), Nationality: Swiss and Panamanian, Marital status: married, 1 child