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Stimulus-Responsive Polymers for Bioseparation

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Abstract: Affinity precipitation uses reversibly water-soluble affinity macroligands, *i.e.* stimulus-responsive polymers bearing one or several affinity ligands, to first capture and then co-precipitate a target molecule. Conventional, polymeric AML tend to suffer from a pronounced heterogeneity in both the structure of the polymer backbone and the affinity constants. In this paper a novel type of homogeneous, oligomeric AML is proposed, which carries one affinity ligand per AML in terminal position. The homogeneity of the AML translates into a very uniform precipitation behavior. Oligomeric AML-precursors prepared by chain or group transfer polymerization show a solubility, which is very similar to that reported previously for polymeric molecules of the same chemistry. Oligomers prepared by anionic polymerization are predominantly isotactic and show some deviations from this behavior. The affinity ligand is coupled to the oligomers *via* a reactive end group (*e.g.* an amino or carboxylic acid group) created during oligomer synthesis. An iminobiotin activated AML-precursor is used to recover avidin from a cell culture supernatant containing 5% FCS. Over 90% of the avidin are recovered in nearly pure form (residual protein contamination below the detection limit). This is one of the first purifications of a protein other than an enzyme by affinity precipitation with high yields. In a second example, the AML-precursor is activated by a single stranded DNA oligomer tag ($(CTT)_7$) and used to purify double stranded DNA molecules by triple helix affinity precipitation.

Keywords: Avidin-biotin system · Cosolute effect · Plasmid DNA · Poly-(N-alkylacrylamide) · Thermoprecipitation · Triple helix affinity

Introduction

Product isolation increasingly becomes a major bottleneck in molecular biotechnology. While considerable efforts are undertaken to use innovative procedures and modern process design software to streamline the actual bioproduction processes for recombinant proteins and more and more also for certain DNA-based bioproducts such gene vaccines, and gene therapeutics, comparatively little progress is being made to improve the downstream process to a similar extent. Since the above-mentioned bioproducts have to be purified from a complex production environment, high-resolution bioseparation techniques are required and most commonly chromatography and bioaffinity-based procedures are used [1]. 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.

Especially early on in the downstream process the rapid removal of water is of major importance. Chromatography and especially affinity chromatography may be used in this context, but may not be ideally suited to the purpose (danger of column fouling). The scale of the volume to be processed, which even in the case of recombinant proteins may easily be in the 10000 l range, may also present a problem. Non-specific precipitation techniques, which are used at large scale, for example, in plasma fractionation [2], are of little use, when the titer of the target molecule is relatively low, *i.e.* in the case of most recombinant bioproducts.

Affinity precipitation is a viable alternative to chromatographic separations, especially in the early stages of a downstream process [3][4]. The method combines the advantages of a highly specific affinity separation with the robustness and scalability of a precipitation technique. Affinity precipitation uses stimulus-responsive affinity macroligands (AML) to first capture and then co-precipitate the target molecule. A typical AML consists of a polymer to which a small affinity ligand is linked. The polymer mediates the response to the stimulus, *e.g.* a change in temperature, the pH or the salt content of the environment; the affinity tag is responsible for highly selective ('biospecific') binding of the target molecule. Once the target molecule has bond in homogeneous solution, the affinity complex is precipitated and thereby removed from the impurities (Fig. 1). Several washing steps may follow; usually by redissolution/reprecipitation of the affinity complex under binding conditions. Afterwards the target molecule is released either *via* direct elution from the precipitate or after redissolution of the complex in a dissociation buffer. Unless regulatory specifications

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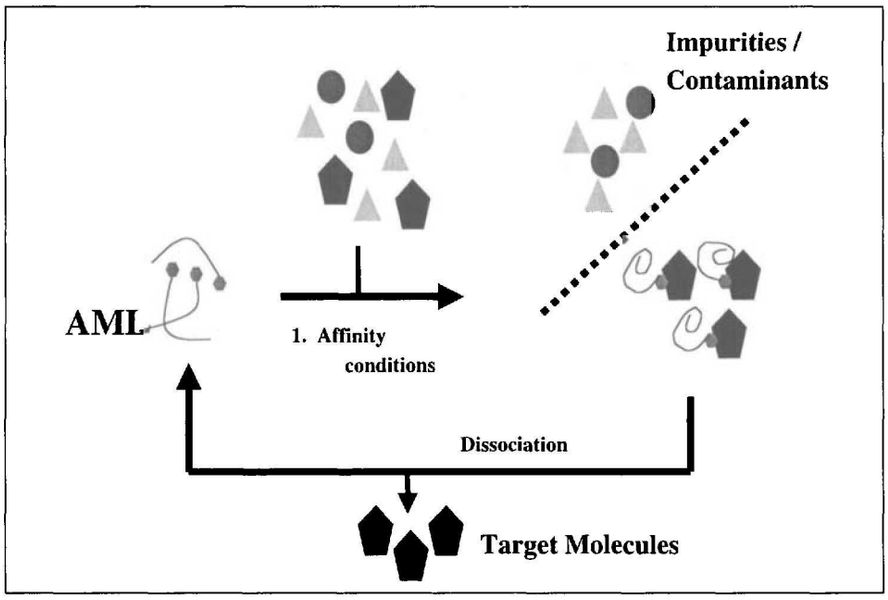

Fig. 1. Schematic presentation of the principle of affinity precipitation.

prevent this, an AML can usually be recycled several times.

Conventional AML suffered from a pronounced heterogeneity of both the polymer backbone and the product-binding site. This made process development rather difficult. We have recently introduced a novel type of thermoresponsive AML [5], which does not suffer from these drawbacks. The polymer is routinely produced by living polymerization (most often chain transfer polymerization) of suitable N-alkylacrylamides [6][7]. The resulting molecules are rather small (< 5000 g/mol) and show little polydispersity (< 1.2). Depending on the synthesis reaction used in a particular case, the molecules carry a reactive end group (*e.g.* carboxylic acid or amino group) to which the affinity tag can be linked by standard coupling chemistry [8].

Experimental

Chemicals and biologicals were obtained from Sigma, Fluka, or Aldrich. The highest available quality was used throughout. Monomers, initiators and solvents were purified prior to use. Polyacrylamide gels, gel electrophoresis equipment and protein assay (Bradford) kit were from Biorad, Hercules, CA, USA and were subsequently used according to the manufacturer's instructions. Gels were silver-stained for detection (Silver Stain Plus kit, Biorad). Spent cell culture supernatants from CHO cell cultivations (DMEM/F12) containing 5% fetal calf serum (FCS) were donated by the laboratory of cellular biotechnology (Prof. F. Wurm), Swiss Federal Institute of Technology at Lausanne.

The anionic and group transfer polymerization of N,N-diethylacrylamide were carried out as described previously [6][10]. Poly-N-alkylacrylamides (Poly-NIPAAm and Poly-DEAAm) with terminal amino group (and carboxylic acid group respectively) were synthesized by radical polymerization in the presence of the chain transfer agent 2-mercaptoethylamine (and mercaptopropionic acid respectively) following a method described previously by Takai *et al.* [7][10]. The activation of the AML precursor Poly-NIPAAm and the addition of the affinity tag iminobiotin was done as described previously [11]. For triple helix affinity precipitation, single stranded oligonucleotides ($(CTT)_7$-DNA) were coupled by carbodiimide coupling to Poly-NIPAAm oligomers carrying a terminal carboxylic acid group.

Affinity precipitation of avidin: Binding took place at pH 10.8. The indicated amount of AML was added to the gently stirred avidin-containing cell culture supernatant. After complete dissolution of the AML, the AML avidin-complex was thermoprecipitated (2 °C above the LCST of the system) and isolated from the supernatant by centrifugation (10000g, 2 °C above the LCST, 5 min). The recovered AML avidin-complex was redissolved in fresh binding buffer (0.1M sodium carbonate buffer pH 10.8) and again precipitated ('washing step', S2). Washing was repeated once (S3). The complex was then dissolved in 0.1M ammonium acetate buffer, pH 4, containing 0.5M NaCl (dissociating buffer) in order to liberate the avidin. The AML was subsequently removed by precipitation (supernatant: S4). The dissociating step was repeated once (supernatant: S5).

The binding buffer for triple helix formation was a 0.2M acetate buffer (pH 4.5) containing 2M NaCl. For THAP, the sample mixture was incubated with 50 mg of the AML in 0.2M acetate buffer (pH 4.5) for 12 h at 4 °C under gentle stirring. Afterwards the affinity complex was thermoprecipitated at room temperature and isolated from the supernatant (binding step, S1) by centrifugation (10000g, 25 °C, 8 min). The recovered complex was suspended in fresh binding buffer and gently stirred at 1 °C. After complete redissolution, the complex was again precipitated ('washing step', S2). The complex was then dissolved in a 0.3M borate buffer (pH 9) containing 0.5mM EDTA in order to liberate target molecule from the AML. The AML was subsequently removed by precipitation (release step, S3) and centrifugation (10000g, 40 °C, 10 min).

For the determination of the critical temperature (cloud points), the optical density of the oligomer solution was monitored as a function of the temperature at 500 nm. Heating rates were 0.5 °C/min. Water was used as a reference. Cloud points were taken at the inflection point in the resulting optical density *versus* temperature curves. The oligomers were further characterized by ^1H NMR and ^{13}C NMR spectra using $CDCl_3$ as solvent and by MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) analysis. Values for the apparent weight average of the molecular mass, *Mw* and the apparent number average of the molecular mass, *Mn*, were calculated from the mass spectra using the following formulas:

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

where M_i is the mass of a given unimolecular oligomer species, while N_i is the number of the molecules of that mass in the preparation. The degree of polydispersity, P, was calculated as:

$$P = M_w/M_n$$

Phase transition enthalpies were determined by high sensitivity differential scanning calorimetry.

Results and Discussion

Solubility Behaviour of the Oligomeric AML-Precursors

A number of thermosensitive, *i.e.* reversibly water-soluble polymers can be found in the literature [12]. These polymers are soluble in cold water, but precipitate rapidly once a critical solution temperature (T_{cr}) has been surpassed. In order to have a critical temperature in the physiological range, our research has concentrated on two structures, namely poly-(N-isopropylacrylamide), Poly-NIPAAm $T_{cr} \approx 34$ °C, and poly-(N,N-diethylacrylamide), Poly-DEAAm $T_{cr} \approx 32$ °C. While Poly-NIPAAm was accessible only by radical (chain transfer) polymerization (telomerization), Poly-DEAAm could also be produced by anionic and group transfer polymerization. The Table compiles some typical data of the compounds produced following the respective synthetic protocols.

The molecular masses of all molecules are relatively low (< 5000 g/mol), which is an advantage for a future AML, since the end group density per gram of AML-precursor and hence the coupling site density for the affinity ligands is high. The polydispersity of the compounds is also low (< 1.2), which translates into a uniform and homogeneous precipitation behavior. Molecules produced by telomerization and group transfer polymerization show a critical solution temperature, a phase transition enthalpy and a general solution behavior, for example also in regard to the effect of cosolutes and cosolvents, which is close to the one reported in the literature for much larger molecules of similar chemistry [7][13–5].

Poly-DEAAm produced by anionic polymerization, on the other hand, shows some interesting deviations from this behavior. The transition enthalpy of the anionic Poly-DEAAm is twice as high, while the actual critical transition temperatures is several degrees higher than in case of the corresponding telomers and group transfer polymers. The effect of co-solutes such as simple salts, which are assumed to exert their influence mainly *via* the water structure and not by direct interaction with the dissolved polymers, is similar for all investigated poly-(N-alkylacrylamides) [16]. On the other hand, in the case of cosolutes such as surfactants respectively amphiphilic molecules in general, *i.e.* molecules which may be assumed to interact with the dissolved oligomers (*e.g.* formation of 'mixed micelles'), distinct differences can be observed between the behavior of the anionic Poly-DEAAm and that of all other investigated poly-N-alkylacrylamides). In these investigations it is generally found that the ability of the anionic Poly-DEAAm to promote hydrophobic interactions is significantly higher [16].

From high-resolution (600 MHz) ^{1}H NMR data it became evident that the anionic Poly-DEAAm is predominately isotactic, while the telomers and group transfer polymers are mainly heterotatic. How this difference affects the solution behavior respectively how the observed differences are related to the tacticity of the molecules, is currently under investigation in our laboratory.

Application of Thermoresponsive AML for Bioseparation

In principle, all of the above-mentioned poly-(N-alkylacrylamides) oligomers constitute possible AML precursors. However, the molecules produced by telomerization have the advantage that each oligomer starts with a reactive group such as a carboxylic acid or an amino group. Affinity ligands may be linked to such groups using coupling chemistries originally established for affinity chromatography and related techniques. In addition, all affinity ligands are linked in an identical manner to the AML. This is different from the situation found in conventional AML, where several affinity ligands are usually distributed in a statistical manner over the polymer backbone. Moreover, ligands located at the polymer end points tend to preserve their affinity to a high degree, since they are more accessible than ligands bond somewhere along the polymer chain. Consequently, the novel oligomeric AML proposed here are characterized by a high and uniform affinity constant. Due to the small size of the AML, a sufficient ligand density is nevertheless reached even though only one affinity ligand is introduced per AML molecule.

In a first application, the novel AML were used for the purification of avidin [11]. The strong affinity ($K_D = 10^{-15}$M) between biotin and avidin has been used countless times throughout the life sciences to securely couple an avidin-carrying species to its biotin-labelled counter part. In our opinion, the avidin/biotin-system represents a possible generic approach to affinity precipitation, since biotin carrying AML can be used to purify avidin-tagged products and *vice versa*. There is perhaps one problem linked to the use of this system for separation procedures like affinity precipitation and that is the remarkable stability of the avidin/biotin-complex, which makes product recovery difficult. Many proteins cannot tolerate the harsh conditions (6M guanidine-HCl, pH 1.5) required to dissociate the complex. This problem can be overcome by replacing biotin with one of its analogues such as 2-iminobiotin. At high pH (> 10), 2-iminobiotin binds tightly and with high specificity to avidin. At lower pH, this interaction is much weaker (*e.g.* $K_D \sim 10^{-3}$ at pH 4). A suitable AML for avidin purification was produced by coupling 2-iminobiotin to an amino-terminated oligomer.

The AML was subsequently used to recover avidin from a cell culture supernatant containing approximately 5% of fetal calf serum. For the recovery the pH of the cell free supernatant was adjusted to 10.8 prior to the addition of the AML. Subsequently, the AML avidin-complex was thermoprecipitated and isolated from the supernatant (S1) by centrifugation. The recovered AML avidin-complex was redissolved in binding buffer and again precipitated ('washing step', S2). This was repeated at least once (supernatant S3). The complex was then dissolved in dissociating buffer in order to liberate the avidin from the AML. The AML was subsequently removed by precipitation (supernatant S4). The dissociating step was usually also repeated once (supernatant S5). The results are summarized in the gel shown in Fig. 2A. Fig. 2B summarizes the avidin content of the various su-

Table. Technical data of thermoresponsive polymers prepared throughout this work.

	Mw	Mn	D	Q_{trans}	LCST	End Group	Structure
A[a]	4500	4000	1.12	44.9 J/g	39.0 °C	butyl	isotactic
G[a]	2090	2380	1.14	20.8 J/g	31.4 °C	carboxyl	heterotactic
T[a]	2650	2000	1.32	17.5 J/g	32.8 °C	amino	heterotactic
N[b]	2700	2300	1.17	16.5 J/g	32.7 °C	amino	heterotactic

[a] Poly-DEAAm, [b] Poly-NIPAAm, A: anionic, G: group transfer, T: chain transfer polymerization (telomerization)

pernants collected during the isolation procedure. More than 90% of the avidin originally present in the cell culture supernatant were recovered in purified form during the first and second release step (S4 and S5). The residual protein contamination of the purified avidin was below the detection limit, and the specific avidin content was enhanced about 14-fold. To our knowledge, this is the first time that the isolation of a biological target molecule other than an enzyme was successfully achieved by affinity precipitation with a significant yield.

The use of affinity precipitation to purify and/or remove DNA presents another interesting option. In a second application, AML bearing single stranded oligonucleotide ligands were created and used to purify double stranded DNA-molecules *via* triple helix affinity interaction (Triple Helix Affinity Precipitation, THAP-process), see Fig. 3 [16]. Triple helical nucleic acid structures were first identified in 1957 by Felsenfeld and Rich [17], only four years after Watson and Crick proposed the DNA double helix. However, the interest in these structures declined rapidly once it had been established that they have no biological function. Recently, it has been recognized that the formation of triple helices could serve as the bases for the design of site specific duplex DNA binding agents (*i.e.* affinity ligands).

In a typical THAP-separation experiment, a mixture (1:1) of double stranded DNA molecules, which either contained the interactive 5'-$(GAA)_7$-3'-sequence (specific or 'Sp' oligo) or which did not contain this sequence (unspecific or 'Up' oligo), was incubated with the AML (Poly-NIPAAm-$(CTT)_7$) in binding buffer. After incubation, the triple helix affinity complex was thermoprecipitated and isolated from the supernatant (S1). The recovered triplex was suspended in fresh binding buffer and again precipitated ('washing step', supernatant S2). The complex was then dissolved in the dissociation buffer to release the bound molecules from the AML. The AML was subsequently removed by precipitation (supernatant S3). A typical result is shown in Fig. 4.

More than 75% of the target molecules (Sp-duplex) were found in the precipitate, compared to less than 10% of the 'contaminant', *i.e.* the Up-duplex. Correspondingly, 80% of the contaminating Up-duplex molecules stayed in the supernatant during the affinity precipitation separation compared to only 20% of the target Sp-duplex molecules. Additional washing steps may conceivably still improve this result.

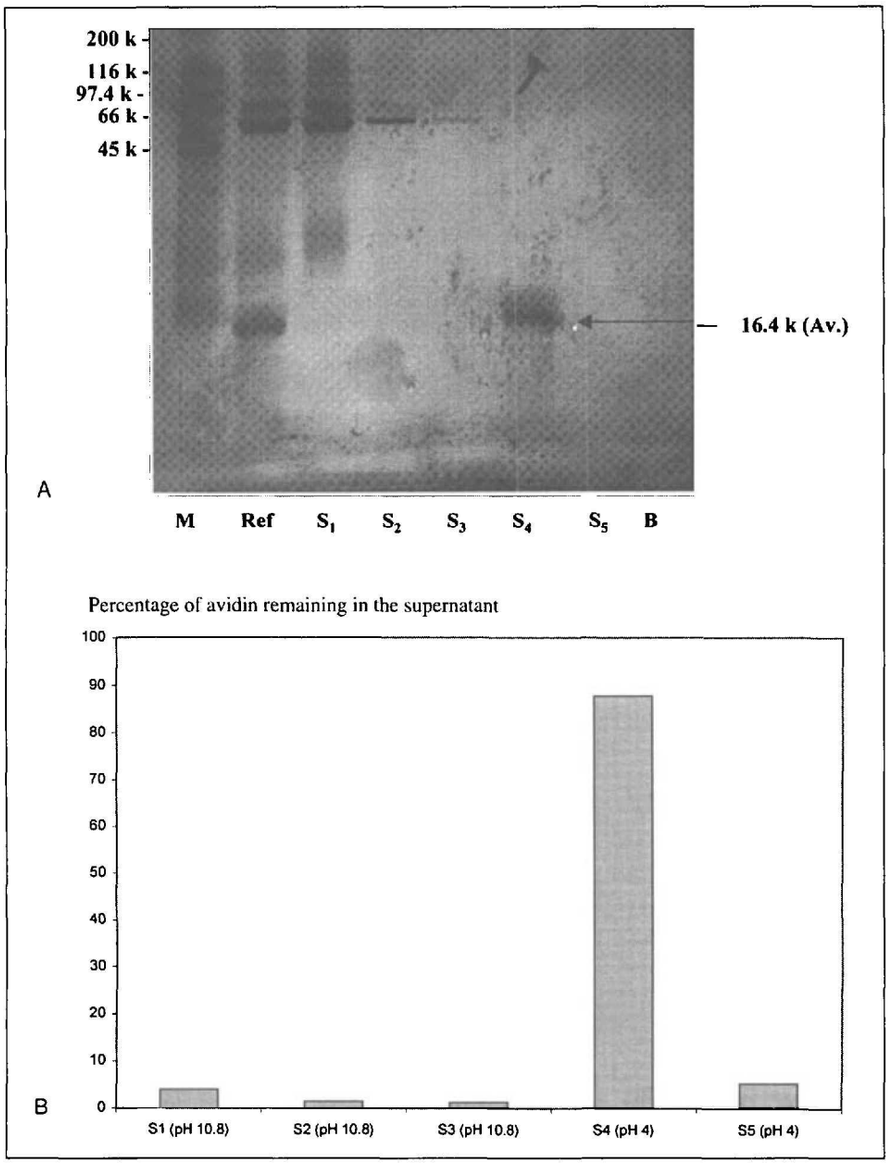

Fig. 2. A: Isolation of avidin from cell culture supernatant; documentation by SDS-PAGE gel (silver stained). M: molecular mass markers, Ref: avidin containing feed, S1: feed after affinity precipitation, S2 and S3: supernatants obtained during precipitation from consecutive volumes of fresh binding buffer (washing steps), S4: first dissociating buffer supernatant (release of avidin), S5: second precipitation of the AML from dissociating buffer, B: buffer sample. B: Avidin content (% of the original amount in the culture supernatant) determined in the various supernatants collected during isolation by affinity precipitation. S1: original culture supernatant after precipitation, S2: first washing step, S3: second washing step, S4: supernatant after release of the target molecule (avidin), S5: supernatant after second precipitation from dissociating buffer.

Conclusions

Affinity precipitation is shown to combine the high performance and the simplicity of a precipitation with the high selectivity of an affinity separation of proteins and DNA. The method is highly specific and efficient and putatively has an excellent scale up potential.

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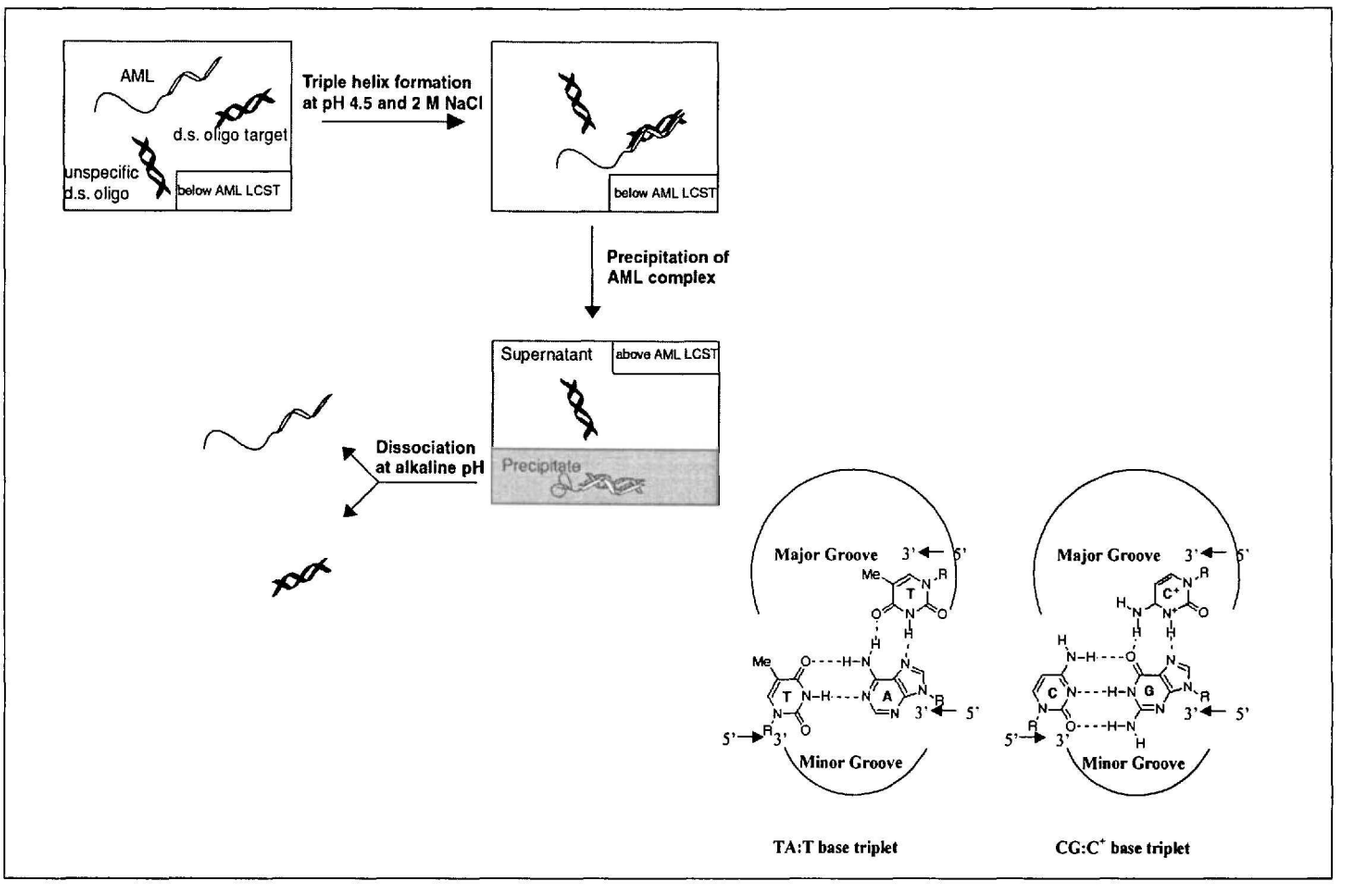

Fig. 3. Principle of Triple Helix Affinity Precipitation (THAP).

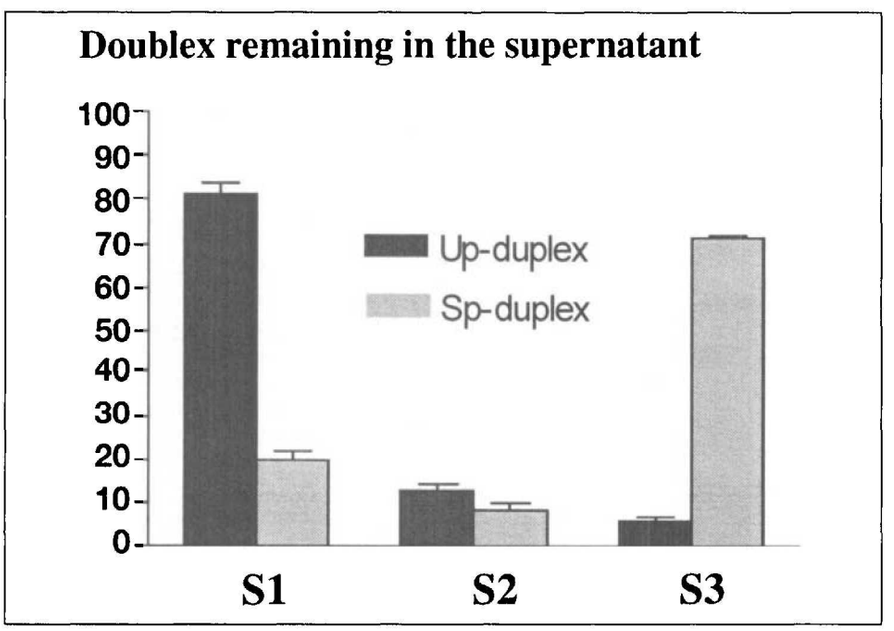

Fig. 4. Content of the target DNA molecule (Sp-duplex) and the contamination (Up-duplex) (% of the original amount in the mixture) determined in the various supernatants collected during separation by affinity precipitation. S1: original mixture after precipitation, S2: washing step, S3: supernatant after release of the bond DNA molecules.

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