Bio-Works

APPLICATION NOTE

Conversion of ion-pairing agents/counter ions

Removal of unwanted ion-pairing agents from peptides and oligonucleotides

Efficient purification of target molecules, especially of therapeutic molecules, is of the utmost importance. Equally important, however, is the removal of residuals, such as ion-pairing agents (IP agents), used in the purification processes. IP agents are used to improve the separation of hydrophilic and polar molecules in reversed phase chromatography (RPC), also known as ion pair chromatography (IPC). Such separation techniques are primarily being used to purify peptides and oligonucleotides. IP agents interact very tightly with the target molecules and are thus difficult to remove. Many different removal strategies have been employed for different combinations of targets and IP agents. Ion exchange chromatography (IEX) is one such method that can be used to facilitate removal of IP agents for both peptides and oligonucleotides. Here the principals, advantages and disadvantages, and operational protocols when applying IEX for this purpose are described.

Ion-pairing agents

Using RPC for the separation of oligonucleotides and many peptides, requires ion-pairing agents (counter ions) in the mobile phases. Without ion-pairing agents it is very challenging to separate hydrophilic molecules since they are not retained efficiently on the stationary phase. Ion-pairing agents associate with charged molecules via electrostatic interactions, thereby modifying their polarity, rendering them inert and thus increasing their affinity to the hydrophobic ligands. The addition of these small opposite charged agents (as ion pairs) when running RPC/IPC is a very valuable tool for so-called “tides” separations. Commonly used ion-pairing agents include trifluoroacetic acid (TFA), triethylamine (TEA), dibutylamine (DBA), tributylamine (TBA), and *e.g.* hexane-sulfonic acids. The negatively charged TFA is frequently used as a counter ion to render cationic peptides inert under acidic conditions, and the positively charged DBA and TBA are often used as counter ions to interact with oligonucleotides under more neutral conditions.

Common ion-pairing agents

- TFA (trifluoracetic acid)
- TEA (triethylamine)
- DBA (dibutylamine)
- TBA (tributylamine)
- Sulfonic acids

Removal of ion-pairing agents

Even though the presence of ion-pairing agents is necessary for optimal separation of polar species, they also typically need to be converted to another more biocompatible ion in subsequent purification steps since they can be toxic and risk interacting with further downstream assays and purifications. The efficiency of this conversion differs depending on the ion-pairing agent present. Counter ions are difficult to remove since they tightly interact with the opposite charges of the target molecules.

There are however many approaches that can be used to remove ion-pairing agents, including chromatographic strategies using IEX and RPC, where either the target molecules or the counter ions can be bound to the resins for exchange of ions (Table 1).

Table 1. Examples of different ion-pairing agents exchange strategies that may be considered.

Strategy	In practice
IEX – binding of target	Works well at reduced flow rates, see a more detailed description in text below.
IEX – binding of IP agents	Difficult to reach equilibrium/binding of IP agents unless done in batch mode.
Desalting	Not possible due to too slow conversion rate.
Replace IP agent with a stronger acid	Works, but a stronger acid than TFA is required which can chemically affect the target molecule and its activity. Limitations of available counter ions for the conversion.
RPC – binding of target	The efficiency of the conversion depends on the target and IP agent. Does not work for more hydrophilic and polar species. TFA can be converted to *e.g.* acetic acid (HAc) when the targets are bound to the stationary phase.

TFA removal from peptides using CIEX

TFA anions are often exchanged to chlorides due to higher biocompatibility of the latter. The most frequently adopted method is to replace TFA counter ions with a stronger acid such as hydrochloric acid (HCl), but these very acidic conditions may damage the peptide. Therefore, other milder conditions are preferred. Two different chromatographic methods can be used; RPC and IEX.

The approach to bind the TFA anions to an AIEX resin has proven to be difficult unless performed in batch adsorption mode under constant gentle stirring. The same goes for cationic ion-pairing agents that are electrostatically bound to oligonucleotides, such as DBA; they cannot bind to the ligands before the target molecules are eluted off from the column. Here we instead focus on the opposite, to capture the target molecule while exchanging the counter ions/ ion-pairing agents on the stationary phase. It is important to remember that the interactions between the cationic target and the negatively charged ligands will require some time, due to the presence of the ion-pairing agents blocking many of the interaction sites on the peptide. Therefore, a slow flow rate must be applied during the loading phase of the target molecule. Also, pH must be lower than the isoelectric point (pI) of the peptide to ensure that the peptide is in a cationic state. The ionic strength must not be too high to facilitate ionic interactions and a final desalting step may be needed after the elution. Many peptides and oligonucleotides require addition of organic solvents, such as acetonitrile or ethanol, to obtain optimal binding and elution conditions.

When the ion-pairing agent conversion is completed, the efficiency of TFA removal must be confirmed. TFA can be assessed by gas chromatography (GC), mass spectrometry (MS), reverse phase HPLC, infrared spectroscopy (IS), titration, NMR or spectroscopy for example.

Protocols for TFA removal using CIEX for peptides with lower pIs

TFA-Acetate conversion for peptides with pI 4.2 – 5.5 using CIEX

- Pack a column with WorkBeads 40S (CIEX).
- Perform a counter ion loading step (3 – 5 CV) of the resin by running a high concentration of acetate, *e.g.* 250-500 mM ammonium acetate (NH_4OAc).
- Equilibrate the column with your binding buffer including acetate using *e.g.* 10 mM NH_4OAc, pH 4.
- Apply the peptide to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Peptide load depends on the sequence (~ 60 – 130 mg peptide/ mL resin).
- Wash the column (~ 10 CV or longer) with binding buffer to ensure conversion of TFA to acetate (can be done at a higher flow rate).
- Elute the target peptide with 250 – 500 mM NH_4OAc, pH 5.6-6 (pH should be above the pI of the peptide).

TFA-Acetate conversion for peptides with pI 3.0 – 5.5 using CIEX

- Pack a column with WorkBeads 40S (CIEX).
- Perform a counter ion loading step (3-5 CV) of the resin by running a high concentration of acetate, *e.g.* 250 – 500 mM NH_4OAc.
- Equilibrate the column with your binding buffer including acetate using 0.1 M HAc.
- Apply the peptide to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Peptide load depends on the sequence (~ 60 – 130 mg peptide/ mL resin).
- Wash the peptide (~ 10 CV or longer) with binding buffer to ensure conversion of TFA to acetate (can be done at a higher flow rate).
- Elute the target peptide with 250 – 500 mM NH_4OAc, pH 5.6 – 6 (pH should be above the pI of the peptide).

TFA-Chloride conversion for peptides with low pI using CIEX

- Pack a column with WorkBeads 40S (CIEX).
- Perform a counter ion loading step (3 – 5 CV) of the resin by running a high concentration of chloride, *e.g.* 1 – 3 M sodium chloride (NaCl).
- Equilibrate the column with your binding buffer including chloride using *e.g.* 10 mM NaCl in a pH below the isoelectric point of your peptide.
- Apply the peptide to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Peptide load depends on the sequence (~ 60 – 130 mg peptide/mL resin).
- Wash the peptide (~ 10 CV or longer) with binding buffer to ensure conversion of TFA to chloride (can be done at a higher flow rate).
- Elute the target peptide with 1 – 3 M NaCl. A divalent salt, such as $CaCl_2$ may be used to increase elution efficiency.

Protocols for TFA removal using CIEX for peptides with higher pIs

TFA-Acetate conversion for peptides with high pI using CIEX

- Pack a column with WorkBeads 40S (CIEX).
- Perform a counter ion loading step (3 – 5 CV) of the resin by running a high concentration of acetate, *e.g.* 1 M NH_4OAc.
- Equilibrate the column with your binding buffer including acetate using *e.g.* 10 mM NH_4OAc, pH 5.
- Apply the peptide to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Peptide load depends on the sequence (~ 60-100 mg peptide/mL resin).
- Wash the peptide (~ 10 CV or longer) with binding buffer to ensure conversion of TFA to acetate (can be done at a higher flow rate).
- Elute the target peptide with 1 M NH_4OAc, pH 5.5.

TFA-Chloride conversion for peptides with high pIs using CIEX

- Pack a column with WorkBeads 40S (CIEX).
- Perform a counter ion loading step (3 – 5 CV) of the resin by running a high concentration of chloride, *e.g.* 1 – 3 M sodium chloride (NaCl).
- Equilibrate the column with your binding buffer including chloride using *e.g.* 10 mM NaCl.
- Apply the peptide to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Peptide load depends on the sequence (~ 60 – 130 mg peptide/mL resin).
- Wash the peptide (~ 10 CV or longer) with binding buffer to ensure conversion of TFA to chloride (can be done at a higher flow rate).
- Elute the target peptide with 1-3 M NaCl. A divalent salt, such as $CaCl_2$ may be used to increase elution efficiency.

Ion pair removal from oligonucleotides using AIEX

Since negatively charged oligonucleotides are highly polar in nature it is essential to use an ion-pairing agent to achieve a good separation on a RPC/IPC resin. Positively charged ion-pairing agents such as TEA, DBA, and TBA are commonly used in IPC for oligonucleotide separations. They interact very tightly with the negative charges on the oligonucleotides and are thus difficult to remove. When using an AIEX resin the same principle applies as for the TFA conversion for peptides; that is to bind the target to the resin and not the counter ions. Oligonucleotides have low pI, therefore it works well to remove the ion-pairing agents under neutral conditions. It is important to use a low flow rate during the exchange phase to promote interactions with the partially blocked sites.

DBA-Sodium conversion for oligonucleotides using AIEX

- Pack a column with WorkBeads 40Q (AIEX).
- Perform a counter ion loading step (3 – 5 CV) of the resin by running a high concentration of chloride, *e.g.* 2 – 3 M NaCl.
- Equilibrate the column with your binding buffer including sodium using *e.g.* 10 mM NaCl.
- Apply the oligonucleotides to the column at a low flow rate (residence time of *e.g.* 5 – 10 minutes). Oligonucleotide load of ~ 40 – 50 mg oligo/mL resin is recommended.
- Wash the oligo (~ 10 CV or longer) with binding buffer to ensure conversion of DBA to sodium (can be done at a higher flow rate).
- Elute the target oligonucleotide with 2 – 3 M NaCl.

* If PS-oligonucleotides are applied, ~10 – 30% acetonitrile might need to be added for solubility and to prevent unspecific hydrophobic interactions to the resin. For smaller elution volumes of thiophosphorylated (PS)-oligonucleotides use NaCl concentrations in the higher range. Best option is to use a chaotropic elution salt, such as NaBr or $NaClO_4$, to reduce hydrophobic interactions for improved elution efficiency.

Important to remember

- Bind the target molecule to the resin ligand, do not bind the IP agent to the resin ligand
- Use a reduced flow rate during the binding phase
- It is important to work with optimal pH & ionic strength
- Absorbance spectra of TFA and acetate overlaps (*i.e.* TFA is difficult to visually detect in acetate buffers)
- Increased ionic strength in the elution buffer leads to smaller elution volumes of the target
- Peptides and oligonucleotides may require addition of organic solvents, such as acetonitrile in the buffers

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