Isoelectric Focusing

By-

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Isoelectric point

- Isoelectric focusing (also known as IEF or electrofocusing) is a technique that separates charged molecules, usually proteins or peptides, on the basis of their isoelectric point (pI) which is the pH at which a molecule has no overall charge.
- The great resolving power of IEF makes it ideal for detection of microheterogeneity in purified proteins.

Points to be considered

- A molecule at a pH that is below its pI will be positively charged (protonated by the *relatively* acidic solution). So, it will migrate towards the negatively charged electrode (the cathode) when an electric field is applied.
- A molecule at a pH that is above its pI will be negatively charged (deprotonated by the *relatively* basic solution). So, it will migrate towards the positively charged electrode (the anode) when an electric field is applied.
- Migration will only happen so long as the molecule is charged, which it won't be at its pI. Remember that in an IEF experiment the molecules are always forced to migrate through an immobilized pH gradient (IPG).

Principle of Isoelectric Focusing

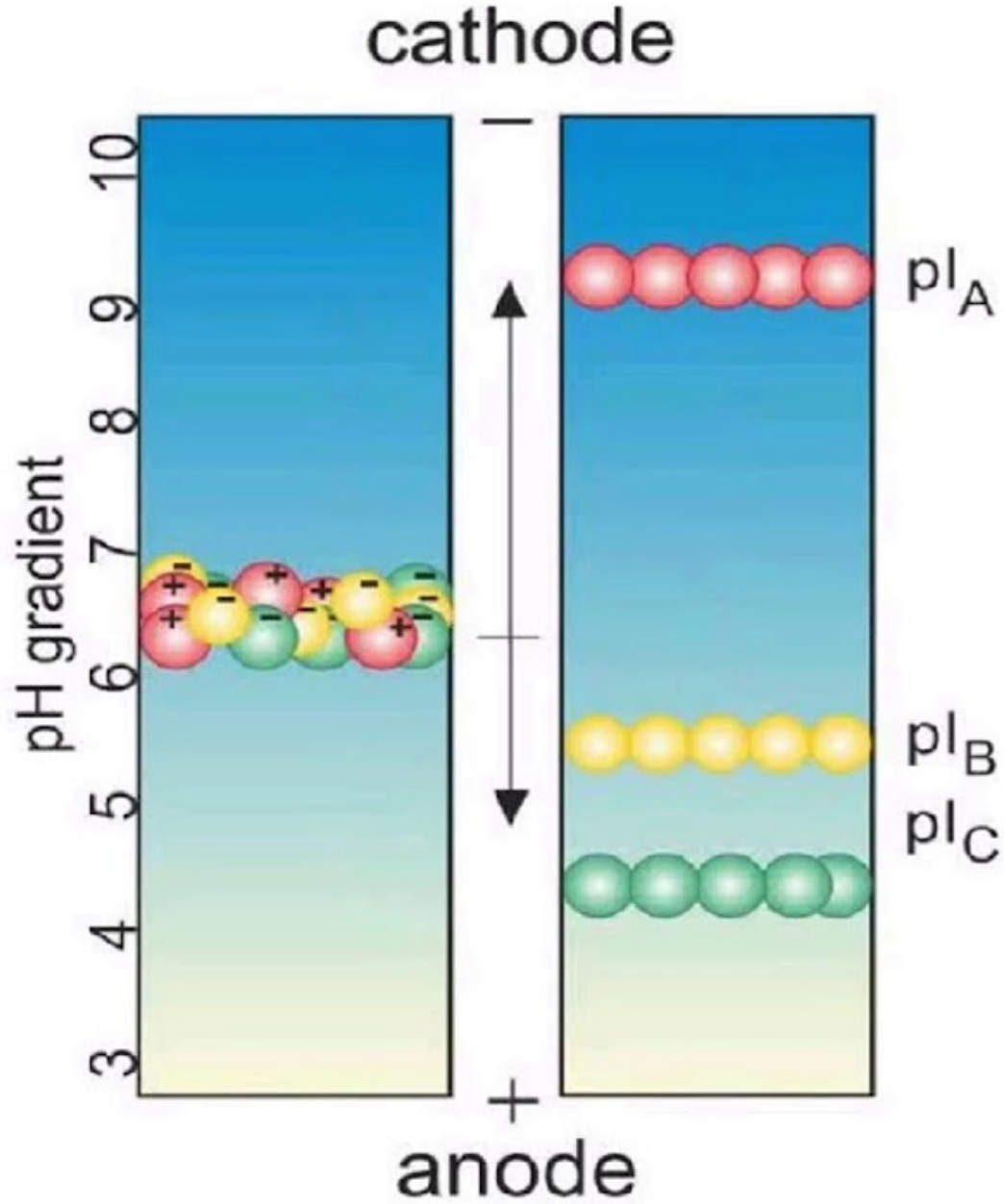
- Isoelectric focusing works because charged molecules will migrate towards regions of opposite charge (the electrodes) when an electric potential is applied to them.
- Force these molecules to migrate through an immobilized pH gradient and they will also migrate towards regions of opposite charge.
- The key point is, once a molecule reaches the area of the immobilized pH gradient that matches its pI, it will no longer be charged.
- Because it’s no longer charged, it no longer experiences an electric attraction to either electrode.
- Do this to a bunch of molecules at the same time and they will all migrate towards their own pI. Hence the name: isoelectric focusing.

= A

= B

= C

amphoteric sample components



Types of IEF

- In order to ensure the high performance of analysis, isoelectric point (pI) standards are needed.
- In addition to classical protein based standards, low molecular weight compounds have been developed and successfully examined in:
  - capillary IEF and
  - IEF-gel electrophoresis

IEF- Gel electrophoresis

- Gel electrophoresis, the common technology for IEF, minimizes convection and introduces an additional gel-sieving effect to separate proteins by size.
- However, it has several disadvantages such as:
  - lengthy analysis time,
  - limited resolution, and
  - difficulty in detection.
- These challenges have been largely overcome by the development of IPG-strips (immobilized pH gradients) for use in high-resolution pI-separation.

IEF- Capillary electrophoresis

- Capillary electrophoresis is a high-resolution approach to separate molecules, based on the pI point, that can be automated.
- Separation is carried out in fused-silica capillaries with internal diameters of 25-75 µM.
- The electrophoresis takes place in free solution and the capillary controls convection currents.
- After focusing is complete, the solutes are pumped out of the capillary.
- UV absorption is the most popular detection method for capillary IEF, but UV induced fluorescence emission is of interest for derivatized proteins.
- Dansyl chloride, fluorescamine, o-phthaldialdehyde, and coumarin moieties are used to increase detection sensitivity.

Key Components

The Gel

- You can prepare your own IEF gels but, because of the requirement for a pH gradient, it's quite long-winded to do so. This in turn invites more errors.
- So, you'll probably just use commercially available IPG strips instead. An IPG strip consists of an acrylamide gel that contains wide pores to prevent any separation of molecules based on their size.
- Various gradient ranges are available.
  - Wider ranges, such as pH 3-10, are suitable for whole proteome analysis, while
  - narrower ranges, such as pH 5-8, are suitable for more specialist applications.

… Key Components

- **The Sample**
- An IEF sample is usually mixed with a solution of carrier ampholytes to assist migration.
- An ampholyte is simply a water-soluble molecule that can act both as an acid and a base depending on pH (just like an amino acid!). The easy movement of the ampholytes through the gel matrix helps the sample molecules to move along the pH gradient with them.
- Again, ampholyte mixtures of a variety of pI ranges are commercially available.

Applications of IEF

1. Combined Use with SDS-PAGE in a 2D-PAGE Experiment

- Isoelectric focusing is traditionally used as the first “dimension” of separation in a 2D-PAGE experiment, in which molecules are first separated by their charge prior to further separation by traditional SDS-PAGE, which is the second dimension of separation.

...Applications of IEF

2. Detection of Post-Translational Protein Modifications

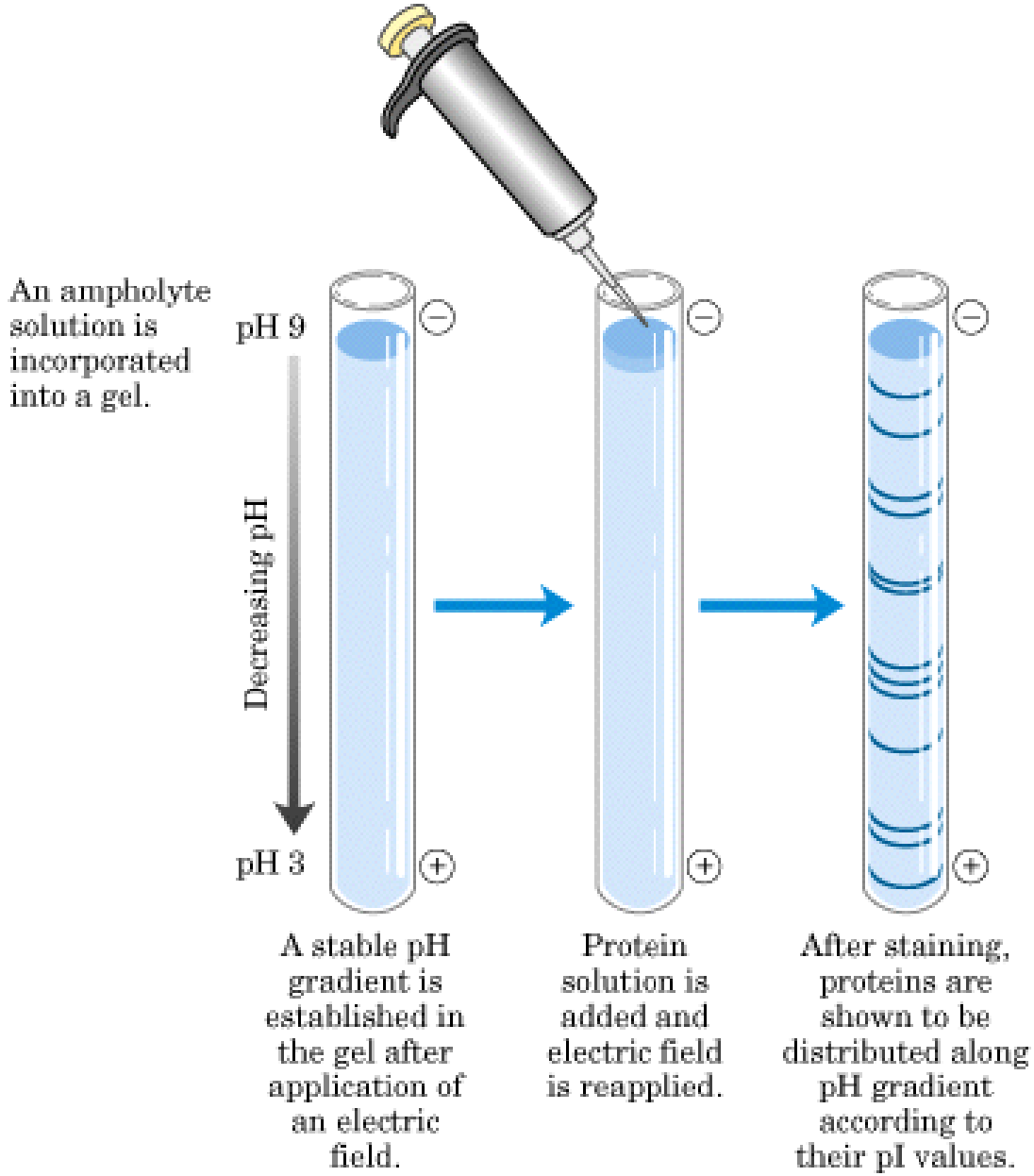
- Isoelectric focusing can also be used to examine post-translation modifications (PTMs) of proteins, which tend to alter their pI.
- For example, phosphorylation, acetylation, and glycosylation (in fact most PTMs) all increase the proportion of negatively charged atoms on a protein (at physiological pH) and therefore lower its pI.
- Of course, PTMs alter the mass of a protein too, but not normally enough to be resolvable by SDS-PAGE.

3. Fractionation of Proteins and Peptides for Mass Spectrometry

- Another use for IEF is for the fractionation of proteins or peptides prior to mass spectrometry.

How IEF Works in Practice

- The IPG strips are rehydrated face down in a denaturing buffer (>6 M urea) with some detergent and usually the carrier ampholytes. For many samples it works better to add the ampholytes with the sample but ultimatately it's a case of trial and error.
- Load the sample. There are two ways to do this: you either include it in the rehydration buffer or you load it in a small plastic cup at the end of the strip. Again, some samples work better with one or other way of loading so give both a try and see what works best for you.
- The strips are then placed in the IEF apparatus and filter paper wicks are placed over the ends of the gel to collect salts and proteins/peptides that are outside of the pI range of the IPG strip.
- Electrodes are placed on top and then you're ready to run!
- The appropriate voltage for an IEF experiment depends greatly on the type of IPG strip used.
- Often enough IEF has to be run overnight.


An ampholyte solution is incorporated into a gel.
pH 9
Decreasing pH
pH 3
A stable pH gradient is established in the gel after application of an electric field.
Protein solution is added and electric field is reapplied.
After staining, proteins are shown to be distributed along pH gradient according to their pI values.