Lecture 3
Isoelectric Focusing
Objectives

• Understand the theory of isoelectric focusing

• Understand how a pH gradient is formed

• Understand immobilised pH gradients (Immobiline)

• Familiarise with IEF experimental techniques

• Set up of an IEF system

• Understand the applications of IEF
Definition of IEF

- An *electrophoretic* process in which proteins are separated by their *isoelectric points (pl)*

- Isoelectric point is the pH at which the protein has zero *net* charge

- Regardless of the point of loading, proteins are “focused” to seek their isoelectric points

- Proteins have a characteristic pl - depending on their amino acid composition - e.g. protein with a lot of acidic side chains - should have a relatively low pl
Isoelectric Focusing - Principle

\[ pH > pI, \text{ proteins are negatively charged;} \]
\[ pH < pI, \text{ proteins are positively charged;} \]
\[ pH = pI, \text{ proteins have no charge} \]

(GE Healthcare 2D-electrophoresis handbook)
Sample separation by IEF

- **Isoelectric focusing (IEF)** separates proteins on the basis of their isoelectric point

- Acid-base properties of amino acids affected by environmental pH

- Glycosylation and phosphorylation affect the isoelectric point of proteins

2D-PAGE: 1st dimension → IEF

(GE Healthcare)
Sialic acid (N-acetylmuramaminic acid)

-ve charged under high pH environment

Oct 2011 SDMBT
Phosphorylation

serine/threonine protein kinases

serine

\[
\begin{align*}
\text{serine} & \quad \text{OH} \quad \text{CH}_2 \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

phosphoserine

\[
\begin{align*}
\text{phosphoserine} & \quad \text{OH} \quad \text{CH}_2 \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

ATP \quad ADP + P_i

threonine

\[
\begin{align*}
\text{threonine} & \quad \text{CH}_3 \quad \text{HC-OH} \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

phosphothreonine

\[
\begin{align*}
\text{phosphothreonine} & \quad \text{CH}_3 \quad \text{HC-OH} \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

ATP \quad ADP + P_i

tyrosine protein kinases

tyrosine

\[
\begin{align*}
\text{tyrosine} & \quad \text{OH} \quad \text{CH}_2 \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

phosphotyrosine

\[
\begin{align*}
\text{phosphotyrosine} & \quad \text{CH}_2 \\
& \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
\end{align*}
\]

80Da

-ve charged

(University of California Irvine)
Theory of Isoelectric Focusing

- The pH gradient is established in an acrylamide gel [see later - 2 ways – carrier ampholytes or immobilised ampholytes]

  e.g. in a carrier ampholyte gel, the anode end of the gel contains phosphoric acid while the cathode contains sodium hydroxide. Therefore the anode will have a low pH while the cathode will have a high pH and a pH gradient will exist between the anode and cathode (for IEF with carrier ampholytes only)

From www.forday.com
To understand how a protein moves in an IEF system, consider a protein with a pI of 8.

If protein is placed on the gel in the pH 6 region, it will become +ve (pH < pI and migrate to the cathode (- electrode attracts + ion)).

As it passes through the gel, eg when it reaches pH 7 region, it becomes less +vely charged (pH not so much <pI) (-electrode does not attract protein so much – protein slows down)
Consider the same protein with a pI of 8.

If protein is placed on the gel in the pH 10 region,

It will become -ve (pH>pI and migrate to the anode (+electrode attracts -ion))

As it passes through the gel, eg when it reaches pH 9 region, it becomes less -vely charged (pH not so much >pI) (+electrode does not attract protein so much – protein slows down)

When reaches the pH 8 region, protein charge =0, -electrode no longer attracts protein, protein stops moving

So no matter where the protein is placed, it will move to the region of the gel where pH=8 after some time as long as there is a voltage across the gel. - focusing – in theory very sharp bands
Formation of a pH gradient

- In order for proteins to seek their isoelectric point, a pH gradient first needs to be established.

- Two types of reagents are added to the acrylamide gel to generate a pH gradient:
  - Carrier ampholytes
  - Immobiline ampholytes
Formation of a pH gradient – carrier ampholytes

Small (300-1000 Da) amphoteric compounds (acidic and basic) Act as buffers

Float around in the gel
Complex mixtures of ampholytes (100’s to 1000’s) each with different pl
eg Commercially available Ampholine, BioLyte etc
When a voltage is applied across a solution containing the mixture of carrier ampholytes

- the carrier ampholytes with the highest pI (and the most positive charge) move toward the cathode.

- the carrier ampholytes with the lowest pI (and the most negative charge) move toward the anode

- other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pH. The result is a continuous pH gradient.
Formation of a pH gradient – immobilised ampholytes

- pH gradient is formed by immobilized ampholytes (ampholytes are covalently bonded to gel – not floating around);

- Gradient drift cannot take place – pH gradient changes over time (>3 hrs) if using carrier ampholytes – cathodic drift – flattening of gradient in neutral region

- Well-defined chemicals give better reproducibility and control of pH gradients;

- Ultraflat pH gradients can easily be prepared (down to 0.01 pH unit/cm) with a concomitant increase in resolution;

  Can have linear or non-linear pH gradient
Immobiline reagents

\[
\text{CH}_2 = \text{CH-C-NH} \quad \text{(GE Healthcare 2-D Electrophoresis Handbook)}
\]

\[
\text{R = weakly acidic or basic buffering group}
\]

(The Alpher, Bethe, Gamow of isoelectric focusing, the alpha-Centaury of electrokinetic methodologies. Part II, Righetti, Electrophoresis 20076, 28, 545–555)

| Table 2. Acidic acrylamido buffers |
|---|---|---|---|
| pK | Formula | Name | \( M_r \) |
| 1.2 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-N.CH\(_3\) | 2-Acrylamido-2-methylpropane sulfonic acid | 207 |
| 3.1 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_3\)-COOH | 2-Acrylamidoglycolic acid | 145 |
| 3.6 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-COOH | N-Acryloyl-glycine | 129 |
| 4.6 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-COOH | 4-Acrylamidobutyric acid | 157 |

(The Alpher, Bethe, Gamow of isoelectric focusing, the alpha-Centaury of electrokinetic methodologies. Part II, Righetti, Electrophoresis 20076, 28, 545–555)

| Table 3. Basic acrylamido buffers |
|---|---|---|---|
| pK | Formula | Name | \( M_r \) |
| 6.2 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-N.CH\(_3\) | 2-Morpholino ethylacrylamide | 184 |
| 7.0 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_3\)-N.CH\(_3\) | 3-Morpholino propylacrylamide | 199 |
| 8.5 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-N.CH\(_3\) | \( N,N\)-Dimethyl aminooethyl acrylamide | 142 |
| 9.3 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_3\)-N.CH\(_3\) | \( N,N\)-Dimethyl aminopropyl acrylamide | 156 |
| 10.3 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-N.C\(_2\)\(_2\) | \( N,N\)-Diethyl aminopropyl acrylamide | 184 |
| >12 | CH\(_2\)=CH-CO-NH-(CH\(_2\))\(_2\)-N.C\(_2\)\(_2\) | \( N,N,N\)-Triethyl aminooethyl acrylamide | 198 |

(The Alpher, Bethe, Gamow of isoelectric focusing, the alpha-Centaury of electrokinetic methodologies. Part II, Righetti, Electrophoresis 20076, 28, 545–555)
Immobiline reagents form part of the IEF gel

Mixtures of different amounts of amine and carboxylic groups result in different pH environments

(GE Healthcare 2D-electrophoresis handbook)
## Immobiline in dry strip format

### Guidelines for choosing Immobiline DryStrip gels

<table>
<thead>
<tr>
<th>pH range</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>Strip length</th>
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</thead>
<tbody>
<tr>
<td>3-5.6 NL</td>
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<td>24 cm</td>
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</tbody>
</table>

**Different pH ranges**

**Different lengths**

(GE Healthcare 2D-electrophoresis handbook) Oct 2011 SDMBT
Choice of pH range

- Broad range pH allow separation of most protein mixtures from prokaryotic and eukaryotic sources

- Narrow range pH allow specific resolution of proteins with a known pI range, especially those that do not resolve well on a broad range gel

- Gel strips with nonlinear (NL) pH ranges allow a more even distribution of proteins along the length of gel to maximise resolution
Immobiline in dry strip format

Linear and non-linear gels

Linear pH gradient over the whole pH range.

Increased resolution between pH 5 to 7 with a non-linear pH gradient.
Experimental Techniques in IEF

Vertical gels – like SDS-PAGE gels

Horizontal gels - see next few slides (slides 21-29)

IEF Strips - used for 2D-gel electrophoresis
IEF – Horizontal gels

Place a few drops of light paraffin oil to adhere the plastic template to the cooling plate and smear it over the surface.
Place the template on the cooling plate. Make sure there are no air bubbles trapped between the cooling plate and the template.
Place a few drops of paraffin oil on the plastic template. Then place the gel on top. Align the gel carefully on the template again ensuring that no air bubbles are trapped between the polyester backing of the gel.
Press the wrapping of the gel to squeeze out air bubbles. You may have to peel off a bit of the plastic packing slightly to see if there are any bubbles. When there are no more bubbles, remove the wrapping entirely.
Carefully place the sample applicators (filter paper 8mmx5mm) using a pair of forceps on the gel along column 4 of the template (middle column) visible through the gel. Once the applicator has been placed on the gel, DO NOT move it.
Load 15 microlitres of sample on the applicator with a micropipette. Load the same amount of standards on the first, middle and last applicators.
Soak the electrode strips in the appropriate buffers (Cathodic strip 1M NaOH; Anodic strip 1M H₃PO₄)
Lay the strips at the designated positions on the gel. Excess lengths should be cut off so that the strip fits within the gel.
Unscrew the electrode holders and slide them until they are over the electrode strips then place the electrode plate over the gel. Place the safety lid back.
IEF – IPG dry strips

- Used for 2D-gel electrophoresis
  – dehydrated gel between two plastic strips
    so gel needs to be rehydrated
- Contains immobilised ampholytes for defined pH ranges
- Different lengths available
- Sample is applied with gel face-down or by cup-loading
**Guidelines for choosing Immobiline DryStrip gels**

<table>
<thead>
<tr>
<th>pH range</th>
<th>2</th>
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<th>8</th>
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<th>12</th>
<th>Strip length</th>
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</tbody>
</table>

**Different pH ranges**

**Different lengths**

(GE Healthcare 2D-electrophoresis handbook) Oct 2011 SDMBT
Choice of length

- As the length increases
  - Loading capacity increases
  - Resolution of proteins increases
  - Number of spots detected increases
  - Focusing time increases
  - Cost effectiveness decreases
## Choice of length

### Illustration: 2D-PAGE of E. coli sample

*Table 14.* Typical operating parameters for Immobiline Drustrip pH 4-7 gels with E. coli extract and analytical load. The number of detectable spots is increased by roughly the same factor as the increase in separation length. The same relationship is true for other pH intervals as well.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>7 cm</th>
<th>11 cm</th>
<th>13 cm</th>
<th>18 cm</th>
<th>24 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time first dimension (h)</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Time second dimension (h)</td>
<td>1.5</td>
<td>2.5</td>
<td>3</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Sample load (µg proteins) — analytical gels</td>
<td>10</td>
<td>25</td>
<td>30</td>
<td>55</td>
<td>90</td>
</tr>
</tbody>
</table>

*(GE Healthcare 2D-electrophoresis handbook)*
Resolution by choice of pH range and length

Coomassie staining

Round 1
Broad pH range for maximal resolution of all proteins

Round 2
Narrow pH range to resolve particular group of proteins

(Kendrick Laboratories)
Resolution by choice of pH range and length

Coomassie staining

Round 2
Narrow pH range to resolve particular group of proteins

(Kendrick Laboratories)
Rehydration of IEF strips

- Dry gel strips need to be rehydrated with rehydration buffer
- This rehydration can be done with the sample

  • Passive rehydration
    Gel strips are put face-down over buffer+sample 12-15 hrs

  • Active rehydration
    Gel strips are put face-down over buffer+sample and a low voltage of 20-120V is applied for 12-15 hrs

If the rehydration is done without the sample, then it is usually passive and the sample added by cup-loading (slide...
Rehydration

1. Add rehydration buffer into strip holder

2. Peel off backing plastic from IPG DryStrip and place **facing down** onto strip holder
Rehydration

3. Add cover fluid

4. Put on strip holder cover and rehydrate for 10-20hrs
Setting up a closed system during IEF

- Cover fluid = paraffin oil/mineral oil

- Cover fluid and strip holder cover prevent evaporation and drying of the IPG DryStrip, which may then lead to burnt gels

- Also protects the DryStrip from the atmosphere (moisture, contaminants and air)
Holders for gel rehydration

Passive

Active

(GE Healthcare) Oct 2011 SDMBT
Cup loading of sample – after the gel has been rehydrated
Cup loading of sample

- Wash rehydrated IPG DryStrip with water
- Place gel on cup loading strip holder **facing up**
- Place electrode pads between IPG DryStrip and electrode (one for each side)
- Place sample cup and test for leakage with rehydration buffer
Cup loading of sample

- Remove rehydration buffer
- Add sample with sample buffer
- As in the rehydration step, cover fluid and strip holder cover used to prevent evaporation and drying of the IPG DryStrip
Components of rehydration and sample buffer

<table>
<thead>
<tr>
<th>Rehydration buffer</th>
<th>Sample buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% IPG buffer</td>
<td>2% IPG buffer</td>
</tr>
<tr>
<td>8M urea</td>
<td>9M urea</td>
</tr>
<tr>
<td>3g/L DTT</td>
<td>10g/L DTT</td>
</tr>
<tr>
<td>20g/L CHAPS</td>
<td>20-40g/L CHAPS</td>
</tr>
</tbody>
</table>

- Bromophenol blue can be added as a tracking dye but does not give endpoint of IEF
Running conditions for IEF

- Per strip: 50 μA, 10,000V typically (depends on length of strip)

- Typically, a IEF program starts at a low voltage to minimise sample aggregation

- Gradually increased through a series of steps to the desired focusing voltage

- Needs to be *empirically determined*, based on recommended settings in protocols
Running conditions for IEF

- A longer focusing time is required for
  - A longer length of DryStrip
  - A narrow range DryStrip
  - Higher load of protein
  - Higher urea/detergent concentrations

- Reproducibility is determined by the time integral of the voltage, \( V_h \).

- \( V_h \) is a different fixed value for each length of DryStrip
### Illustration: Settings for 11cm and 18cm DryStrip

#### 11-cm strips

<table>
<thead>
<tr>
<th>pH intervals</th>
<th>Step Voltage mode</th>
<th>Voltage (V)</th>
<th>Time (h:min)</th>
<th>kWh</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–11 NL</td>
<td>1 Step and Hold*</td>
<td>500</td>
<td>1:00</td>
<td>0.5</td>
</tr>
<tr>
<td>3–10</td>
<td>2 Gradient</td>
<td>1000</td>
<td>1:00</td>
<td>0.8</td>
</tr>
<tr>
<td>6–11</td>
<td>3 Gradient</td>
<td>6000</td>
<td>2:00</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>4 Step and Hold</td>
<td>6000</td>
<td>0:10–0:40</td>
<td>0.7–3.7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>4:05–4:40</td>
<td>9.0–12.0</td>
</tr>
</tbody>
</table>

#### 18-cm strips

<table>
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<tr>
<th>pH intervals</th>
<th>Step Voltage mode</th>
<th>Voltage (V)</th>
<th>Time (h:min)</th>
<th>Volt-hours kWh</th>
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</thead>
<tbody>
<tr>
<td>3–10</td>
<td>1 Step and Hold</td>
<td>500</td>
<td>1:00</td>
<td>0.5</td>
</tr>
<tr>
<td>3–11 NL</td>
<td>2 Gradient*</td>
<td>1000</td>
<td>1:00 (8:00)*</td>
<td>0.8 (6.0)</td>
</tr>
<tr>
<td>6–11</td>
<td>3a Gradient</td>
<td>8000</td>
<td>3:00</td>
<td>13.5</td>
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<tr>
<td></td>
<td>4a Step and Hold</td>
<td>8000</td>
<td>0:46–1:30</td>
<td>6.2–12.2</td>
</tr>
<tr>
<td></td>
<td>3b Gradient</td>
<td>10000</td>
<td>3:00</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>4b Step and Hold</td>
<td>10000</td>
<td>0:20–0:55</td>
<td>3.2–9.2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>21.0–27.0</td>
</tr>
</tbody>
</table>

Gradual increase
Cleanliness during IEF process

- “The greatest threat is yourself”

- Dry skin and hair introduce **keratin** into IEF process

- Clean all equipment with neutral pH detergent to remove any traces of proteins
Processing after the IEF run

If just doing IEF
- stain with crystal violet or zinc imidazole (IPG strips)
- fix with trichloroacetic acid (horizontal gels) and stain with Coomassie or silver staining (like SDS-PAGE gels)

If doing 2D-gel electrophoresis
- strips can be stored at -80°C for up to several months if not used immediately for the second dimension
- when ready to do second dimension, must be equilibrated
Equilibration

- Before an IPG DryStrip can be used in SDS-PAGE, it has to be immersed in buffer containing SDS to allow the focused proteins to interact with SDS.

- Long equilibration times (10-15min) plus the use of urea and glycerol improves protein transfer to SDS-PAGE.
Equilibration of IEF

- Allow SDS to interact with proteins
- Add bromophenol blue as tracking dye
- Reduce disulphide bonding
Equilibration

- IPG DryStrips are equilibrated on a shaking platform successively with two different equilibration buffers
## Equilibration

<table>
<thead>
<tr>
<th>First equilibration</th>
<th>Second equilibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl (pH 8.8)</td>
<td>50 mM Tris-HCl (pH 8.8)</td>
</tr>
<tr>
<td>2% w/v SDS,</td>
<td>2% w/v SDS</td>
</tr>
<tr>
<td>1% w/v DTT</td>
<td>1% w/v IAA</td>
</tr>
<tr>
<td>6 M urea</td>
<td>6 M urea</td>
</tr>
<tr>
<td>30% w/v glycerol</td>
<td>30% w/v glycerol</td>
</tr>
</tbody>
</table>

- Bromophenol blue is added as a tracking dye to give the endpoint for SDS-PAGE