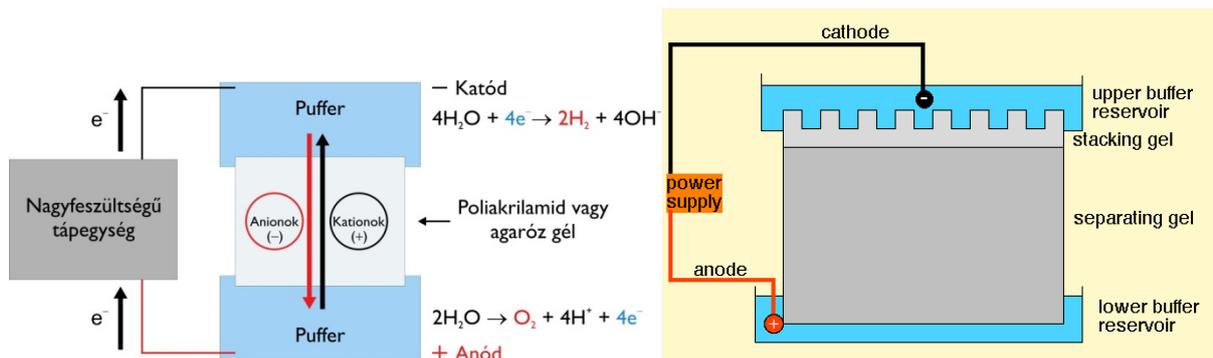


## Detection of proteins by lithium dodecyl sulphate polyacrylamide gel electrophoresis

During electrophoretic measurements a mixture of compounds in solution is taken into a chamber, two electrodes are joined to the ends and the particles that bear charge (proteins, nucleic acid) will move to the electrodes by the effect of electric field. Particles move by different speed according to their charge and size, therefore they are separated. One can distinguish free flow electrophoresis, gel electrophoresis, capillary electrophoresis, isoelectric focusing.

In this practice we will do gel electrophoresis. Both ends of the gel is immersed into buffers, metal electrodes are in the buffers. Applying voltage difference by a proper equipment the ions of the sample and buffer in the gel will conduct the current, in the metallic fibers electrons move from the anode to the equipment and from the voltage generator to the cathode, the electric circuit is closed. Electrophoresis is incomplete electrolysis, so we do not wait for the samples to reach to the electrodes, but the solvent water is cleaved by the effect of electric current. On the anode always oxidation happens, oxygen gas is produced from water, oxidation number of oxygen is increased from -2 to zero. On the cathode always reduction happens, +1 oxidation number H in H<sub>2</sub>O takes up electron to become hydrogen gas.

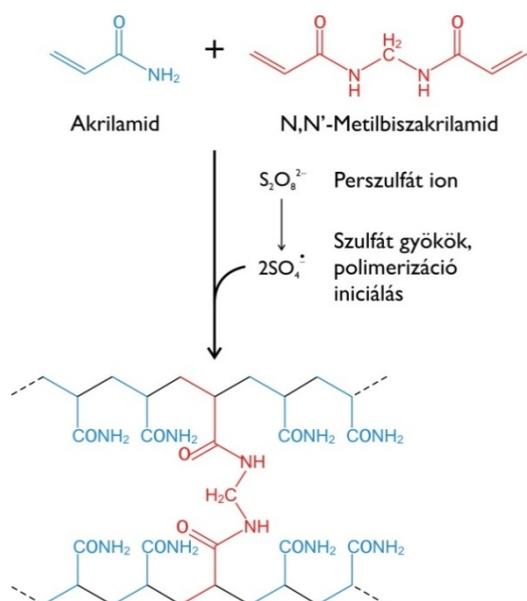


Driving force is the electric, rather electrostatic force that effects on the particles having charge. This force is directly proportional to the charge of the particle and to the electric field. Electric field means the potential difference between the electrodes in case of unit distance.

$$F_e =$$

$$F_e = F_f$$

that is

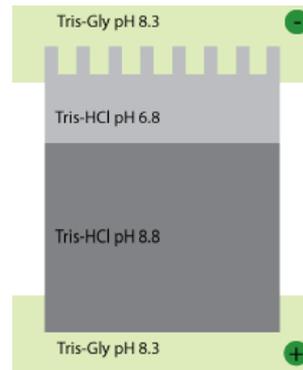


Several types of gels exist: in homogenous gel the pore sizes are more or less the same, in gradient gel the pore size is changed continuously, in discontinuous gel one can find two different pore size gel. The upper gel is the stacking gel having big pore size, its pH is 6.8 and it has no sieving effect for the sample molecules. The larger part is the separating gel or other name is running gel which pore size is smaller, its sieving effect will determine the speed of the migration. Its pH is 8.8-9. Samples are taken to the wells on the gel, wells are formed by a comb that is inserted during solidification and eliminated before electrophoresis. We add bromophenol blue tracking dye to the samples. This molecule has small molecular mass and big negative charge therefore will migrate with the higher velocity toward anode than the samples and tracks the progress of electrophoresis.

#### Buffer

To set the pH we can apply TRIS chloride buffer that contains tris-hydroxymethyl-aminomethane and its hydrochloric salt. Chloride ion is always negative, hydrochloric acid is surely a strong acid, in solution it is completely dissociated at any pH and dilution, therefore chloride ion is the quickest. The other changeable charged particle can be glycine amino acid. The pK value of the carboxyl group is 2.34 and pK of amino group is 9.6. When  $\text{pH} = \text{pK}_1$  the completely protonated +1 charged glycine and the zwitter-ionic neutral forms are found in 50-50%. When  $\text{pH} = \text{pK}_2$  the zwitter-ionic form and the -1 charged glycinate anion are found in the same amount. The isoelectric point (pI) is the mathematical mean of the two pK values, that is 5.87. At this pH the zwitter-ionic net neutral form dominates. The pH of the stacking gel is near to the isoelectric point of the glycine, so small amount of negative and big amount of net neutral forms are present, the driving effect of the electric field is increased for the sample proteins, the voltage is constant, the small amount of the anions in the buffer mean local increase of the resistance. It accelerates the migration of the sample proteins, they enter at the same time to the separating gel. In the running gel high amount of glycine is dissociated because of the alkaline pH, its movement is speeded up, sample proteins retard and can migrate through the pores with different velocity according to their charge and size.

3. neg. glycinate (small amount) or MOPS anion
2. sample proteins
1. Cl<sup>-</sup> and tracking dye

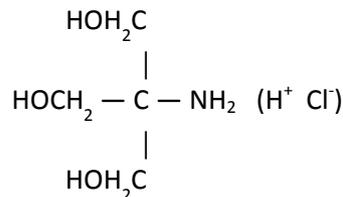
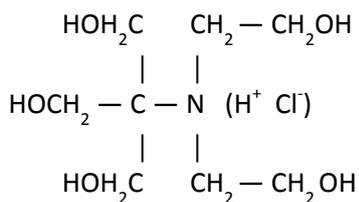


4. sample proteins
3. tracking dye (bromophenol blue)
2. many glycinate anions or MOPS anion
1. Cl<sup>-</sup>

In today's experiment bis-tris is used instead of TRIS, and MOPS is the trailing ion instead of glycine in the running buffer, but the theory is the same as the above mentioned. The always negatively charged chloride ion is the leading ion, the charge of MOPS [3-(N-morpholino)propanesulfonic acid] depends on the pH, it is partially ionized, its pK is 7.2. The pH of gel buffer is 6.4 and pH of running buffer is around 7.5, consequently separation of proteins by electrophoresis is done at neutral pH. It means mild condition, this method can be used for the separation of sensitive proteins. Gels are more stable in neutral pH.

TRIS and its chloride salt

Bis-tris and its hydrochloric acid salt



## Native PAGE

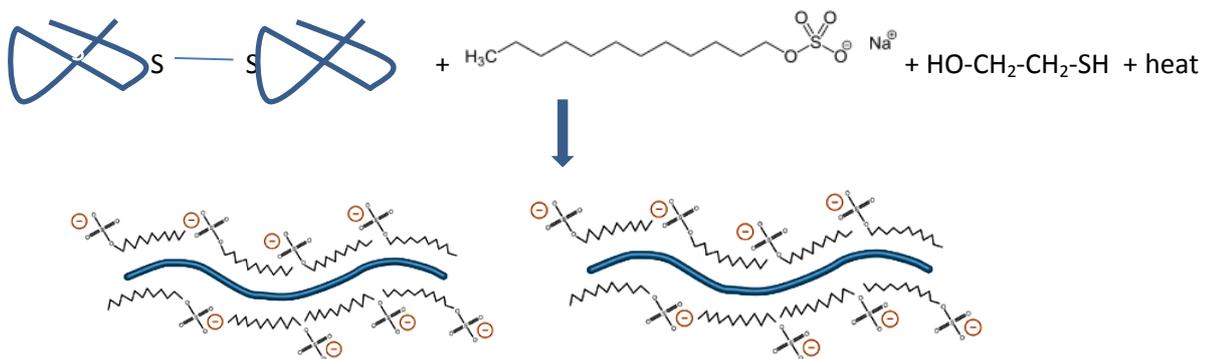
Among the amino acid side chains of proteins there are protonated-deprotonated, carboxyl group of Asp and Glu are acidic, His, Lys and Arg are basic, SH of Cys and phenol of Tyr are weakly acidic. The whole charge of the protein – besides the amino acid composition - depends on the pH of the medium. In mild alkaline condition most proteins have net negative charge, therefore they migrate toward the cathode. When electrophoresis is done at mild condition, the not too sensitive proteins keep their native conformation. Dimers, multimers are not separated, so the quaternary structure is maintained in the non-denaturing condition, at low temperature and in proper buffer. After separation in the gel (in situ) the proteins can be detected, in case of enzymes they can catalyze reactions that lead to coloured compound. One of the biochemistry practice in the second semester is the separation of LDH isoenzymes by PAGE, when NADH – formed during oxidation of lactate – donates H atoms through phenazin metasulphate to tetrazolium blue dye, the appearing blue band shows the localization of lactate dehydrogenase tetramer in the gel.

PAGE is suitable to check the purity of the result during/after protein purification, whether only one component is present (although the aggregation of proteins can be misleading), or several bands show the impurities (or the separation of subunits in the improper condition). Characteristically

during this separation the enzyme remains together with its inhibitor, the receptor with its ligand etc.

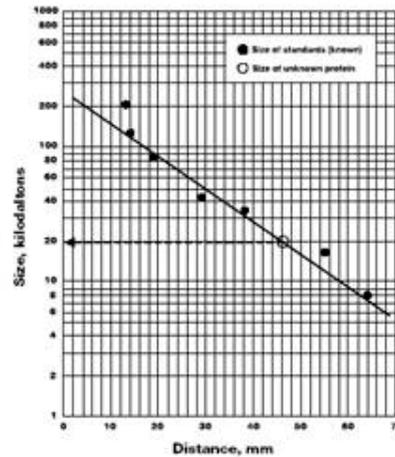
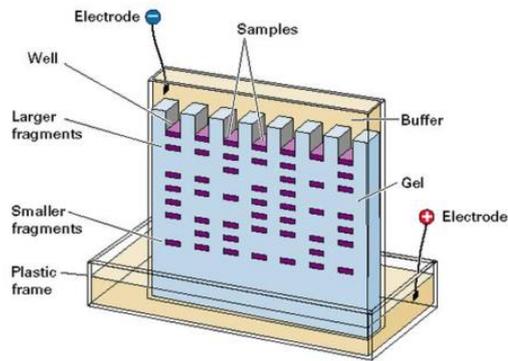
#### SDS-PAGE, LDS-PAGE

During another kind of gel electrophoresis the SDS or LDS anionic detergents, mercaptoethanol or bisulphite SH-reagent and heat are applied together to denature proteins. In the amphipathic detergent molecule the hydrophilic and hydrophobic parts are separated in space.



Sodium or lithium salt of lauryl (=dodecyl) sulphate disrupts the London-type dispersion forces in the inner part of the proteins, secondary, tertiary and quaternary structure of the proteins are broken, long apolar chain of the detergent can join to the unfolded protein, about one SDS or LDS molecule onto two amino acids. So the own charge of the protein becomes negligible, strongly negatively charged complex is formed and the denatured protein is kept in solution because of the charge repulsion and hydrate shell formation. The detergent can dissolve the proteins from membranes. The longer the protein, the bigger amount of detergent can bind to it, the more negative charge will it gain to migrate to the positive electrode, on the contrary the sieving effect of the gel becomes bigger. This retarding effect will determine the speed of the migration. Mercaptoethanol or bisulphite reduces the disulfide bonds between the cysteins, the originally three dimensional molecule becomes straight, the subunits are separated. High temperature accelerates denaturation.

The advantage of the gel that is used today (NuPAGE Bis-Tris Gel) is that cysteins (pK of SH groups 8-9) can not form disulfide bonds at around neutral pH during running, it would happen only in weak alkaline condition during traditional method, and because the quickly moving sodium bisulphite ensures the reducing condition in the gel, opposite to mercaptoethanol which is slower than the proteins. At neutral pH the acrylamide gel is more stable.



We take the samples to the wells on the top of vertical gels. Smaller particles can migrate with higher velocity because of the sieving effect of the gel. The ten based logarithm of the molecular weight of the proteins is inverse proportional to the running distance. Applying mixture of known molecular weight proteins, so called standard together with the sample proteins, one can read the unknown molecular weight comparing to the ladder. This method is proper to determine the purification of proteins.

#### Staining of samples to become visible

Pure proteins and nucleic acids are colourless. To become visible, different methods are used. Dyes that bind aspecifically to every protein is applied frequently. Such dyes are Coomassie Brilliant Blue, Acidic Fast Green, Amido Black, silver bromide. Antibody can be bound to the protein that reacts with another antibody, to which an enzyme is bound, when we add the substrate of this enzyme (e.g. horseradish peroxidase) a coloured, or fluorescent, most often luminescent product is formed. This kind of staining is usual in case of blotting, therefore the name of this method is immunoblot. (Blotting means that from the gel the samples are transferred to another carrier, e. g. to nitrocellulose paper by the effect of electric field in buffer. The method is Southern blot if DNA is transferred, Northern blot in case of RNA transfer and Western blot when proteins are transferred. The pattern remains the same on the nitrocellulose as was in the gel.) There are fluorescent dyes. Ethidium bromide is a polyaromatic intercalating (therefore mutagenic, carcinogenic, terratogenic) compound used to detect DNA, when it is illuminated by UV light it will emit visible light. If radioactive element is built into the protein or nucleic acid ( $^{32}\text{P}$ ), dark band shows the position of sample on x-ray film.

#### Performance of the experiment

First we put together the parts of the electrophoretic equipment and insert the gel into it. We have to use latex gloves because the acrylamide monomers are dangerous. The sticky paper should be removed, through the holes that become free will the gel touch with the buffer. The lower plexi plate looks toward the inner part, we will take the samples there after elimination of gel comb.

Composition of NuPAGE MOPS buffer: 50 mM MOPS [3-(N-morpholino)propanesulfonic acid], 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7 . We make 20-fold dilution of the running buffer , we mix 50 ml running buffer plus 950 ml distilled water in a measuring cylinder. Water must be added slowly to prevent much bubble formation. About 600 ml running buffer has to poured to the lower chamber and about 200 ml to the upper chamber, but the two buffer must not flow together. This gel (NuPAGE® Bis-Tris Gel) and buffer is suitable to separate proteins that have molecular weight between 14-200 kDa.

Preparation of samples into numbered Eppendorf-tubes by the practice teacher:

|                                          | 1  | 2 | 3  | 4 |
|------------------------------------------|----|---|----|---|
| bovine serum albumin (BSA) $\mu$ l       | 2  |   |    |   |
| carbonic anhydrase (CA) $\mu$ l          |    | 7 |    |   |
| cytochrome c (cyt c) $\mu$ l             |    |   | 10 |   |
| alcohol dehydrogenase (ADH) $\mu$ l      |    |   |    | 4 |
| LDS buffer: LDS + dye $\mu$ l            | 5  | 5 | 5  | 5 |
| NuPAGE reducing agent (SH-reag.) $\mu$ l | 2  | 2 | 2  | 2 |
| dest. water                              | 11 | 6 | 3  | 9 |

We incubate the Eppendorf-tubes for 10 min at 70 °C in water bath to denature sample proteins.

We pipet 20-20  $\mu$ l samples - that contain about 20  $\mu$ g proteins - into the gel wells and 10  $\mu$ l molecular weight standard into one well.

We put the lid to its proper place onto the equipment. We apply 175 V constant voltage and 30-40-mA current for one hour. The current decreases during running.

Finishing the running the electrophoretic equipment has to be switched off, electrodes should be taken away, gel is taken out, plastic slabs must be cracked at the edges by gel cutting knife. We cut the pockets from the gel. We cautiously plug the knife under the gel without tearing it then we transfer the gel to the staining bowl .

Washing the gel, elimination of nonpolymerized remnant of materials and of buffer components, is done by repeating the following process 3 times (using gloves):

we pour distilled water onto the gel

boiling in micro wave oven for 1 min at 1000 W

rocking for 1 min by Mini Rocker

pouring out the water to the sink, the gel to remain in the bowl.

Staining the gel

SimplyBlueSafeStain solution contains Coomassie Brilliant Blue G250 dye, 25 ml dye should be poured to the gel and boiled in micro wave oven for 25 sec (no more!). Stain is washed to sink. Then we wash the gel with distilled water and rock it for 10 min to wash out the unbound dye.

Reading the molecular weight of the sample protein to compare with the standard ladder:

Molecular weights of the sample proteins:

BSA: 66 kDa, carbonic anhydrase: 29 kDa, cytochrome c: 12.4 kDa, alcohol dehydrogenase: 150 kDa

**Figure 1—Novex® Sharp Pre-Stained Protein Standard.**

