In order to collect the entire gradient in a series of fractions, several methods may be applied. The simplest is to pierce the bottom of the tube with a needle, and collect the gradient as it drops out. Another method is to pump the gradient from the bottom of the tube with a narrow capillary tube. However, this method is not recommended because of the potential to disturb the gradient and resulting loss of resolution.

See also: **III/Proteins:** Capillary Electrophoresis; Crystallization; Electrophoresis; Field Flow Fractionation; High-Speed Countercurrent Chromatography; Ion Exchange.

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Crystallization

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Introduction

The first protein crystals described in the literature were obtained by Hunefeld in 1840. Hunefeld observed hemoglobin crystals after slow drying of blood pressed between two slides of glass. It is remarkable that this first result demonstrated the basic principle used today, that protein crystals similar to inorganic crystals may be produced by concentration of a protein in solution through slow dehydration. Throughout the history of protein crystal growth, the rationale for protein crystallization has been, firstly, separation of proteins from complex extracts, and then, starting in the 1930s, as purification as determination of the three-dimensional structure of protein molecules.

Knowledge about the three-dimensional structure is necessary to better understand the functions of protein molecules in living systems and plants. Three-dimensional structure can be determined by X-ray diffraction. For X-ray diffraction, good quality protein crystals of appropriate sizes are required. Crystal sizes in each direction should be at least 0.1 mm, if using a strong beam of synchrotron radiation, or at least 0.3 mm for conventional sources of X-rays.

Protein molecules in the crystalline state are more stable than in solution. Therefore, crystallized proteins are more stable against denaturation and may be preserved for a significantly longer period of time than in solution. That is the reason that protein crystallization is often directed as much on preservation as on separation and purification.

This article comprises a brief description of general principles of protein crystal growth and a description of various techniques of protein crystallization with the emphasis on methods using a small amount of a crystallizing solution, from about 1 to 20 μL. The consumption of small amounts of protein is of value, since screening and optimization tests of determination of crystallization conditions typically require many portions of protein solution.

General Principles of Protein Crystallization

Intermolecular Interaction

To crystallize a protein it should be first of all dissolved to give a solution where the protein molecules become close one to another to create a nucleus that grows into a crystal.

An essential feature of protein solutions, associated with the complex structure and large size of protein molecules, is that the molecules may be charged by electric charges, of the same polarity, resulting in long-range electrostatic repulsion. This peculiarity is mediated by the ability of macromolecules to acquire protons from a solution or give up protons into the solution depending on the pH. The charge value of

protein molecules increases with the difference between pH of the solution and pH in the isoelectric point, pI, of the solution. At pH = pI, molecules become neutral, and accordingly, the long-range repulsion is absent. For most proteins pI is in the range 4.5–6.0.

At short distances van der Waals forces of attraction act between molecules. Competition between repulsion and attraction determines the state of protein molecules in solution. If the repulsion dominates, molecules remain apart in solution, protein is dissolved and no clusters or crystals are created. If attractive forces dominate, the molecules gather into clusters, that may form nuclei to yield crystals. For creation of well-ordered nuclei the attractive forces should be strong enough to provide slow clusterization but not so strong as to impair the formation of the crystal structure.

The Coulombic repulsive forces are affected usually by an addition to the protein solution of buffers, salts, precipitants and other additives. Buffers influence acidity; the pH of the solution alters the difference between pH and pI. This in turn changes charge values of protein molecules and accordingly the long-range repulsion. There are various buffers providing different pH: 0.1 M sodium acetate, pH = 4.6, 0.1 M trisodium citrate dehydrate, pH = 5.6, 0.1 M sodium cacodylate, pH = 6.5, 0.1 M HEPES, pH = 7.5, 0.1 M tris hydrochloride, pH = 8.5, etc.

Salts added to a protein solution may screen the repulsive interaction between the molecules. For instance, a 0.1–0.2 M aqueous solution of sodium chloride essentially shields the repulsive electrostatic forces. Precipitants such as polyethylene glycol (PEG) of various molecular weights, isopropanol and sodium formate, decrease the solubility of protein, initiating creation of clusters or nuclei.

Nucleation

Nucleation is initiated by fluctuation of density in a system of atoms or molecules. Crystallization in protein solutions is initiated by protein concentration fluctuation in a solvent. In regions of higher concentration, molecules are associated in clusters because of smaller intermolecular distances thus increasing attractive forces. Generally, clusters may be stable or metastable.

Reduction of repulsive interaction between protein molecules is a necessary condition of nucleation peculiar to proteins and other macromolecular substances. But it is still not enough to create a stable nucleus which becomes a seed for crystal growth. The mutual position of protein molecules should correspond to a minimal energy to form thermodynamically stable nuclei.

Thermodynamic free energy of a cluster in solution consists of two parts: volume energy, ΔG_v and surface energy, ΔG_s. The volume energy is negative, so that it stabilizes the cluster, but surface energy is positive tending to make the cluster unstable. Competition between these two parts of the overall free energy determines the stability of clusters. The total free energy of a cluster, $\Delta G = \Delta G_v + \Delta G_s$ depends on its size, r, so that $\Delta G = \Delta G(r)$. This dependence reveals a maximum at a critical size, $r = r_c$. At sizes larger than r_c clusters become a stable nucleus capable to grow as a crystal. At sizes less than the critical size, clusters tend to dissolve, since they are energetically unstable.

The critical nucleus size, r_c, decreases with an increase of the supersaturation of protein solution $\beta = c/c_{sat}$, since $\Delta G_v \sim -\ln \beta$ and accordingly $r_c \sim 1/\ln \beta$. Here c is the concentration of protein in solution and c_{sat} is the concentration of the protein in saturated solution. In other words, c_{sat} is the solubility of the protein. Consequently, nucleation may occur only at $\beta > 1$, i.e. at protein concentration, c larger than the concentration of saturated solution, c_{sat}. This condition indicates also that the chemical potential of a cluster is lower than the chemical potential of the solution, and accordingly the volume energy ΔG_v is negative. An increase of the supersaturation, β also reduces the maximum of the total free energy, $\Delta G(r_c)$, which should be overcome to create a stable nucleus, because $\Delta G(r_c) \sim 1/(\ln \beta)^2$.

Nucleation is affected by many other parameters, such as relative specific surface energy of protein crystals in solutions, temperature, mobility and substructure of protein molecules, impurities, rate of supersaturation, etc. That is the reason that nucleation of proteins is an important step in protein crystallization, which often requires many screening experiments.

Crystal Growth

A zone of lower concentration is formed around a nucleus after it starts to grow, consuming surrounding molecules. The difference between the protein concentration in the bulk of the solution and in the depletion zone becomes a driving force, transporting the protein molecules from solution to the growing crystal. This mass transport is realized generally by diffusion and convection due to gradients of protein concentration. Slow transport of the molecules is required to yield good quality crystals. This is achieved at low supersaturation and low rate of dewatering of the protein solution and by crystallization at lower temperatures, ~4-6°C.

Mechanisms of attachment of protein molecules to the lattice of growing crystals have been investigated

intensively in recent years with the atomic force microscope. It was demonstrated by Malkin *et al.* with several proteins, lysozyme, thaumatin, canavalin, catalase and apoferritin, that macromolecules grow by all surface integration mechanisms involved in the crystallization of small molecules. The protein crystals reveal growth on screw dislocations and by two- and three-dimensional nucleation.

A screw dislocation generates steps that propagate along the crystal surface. The steps contain kinks at the crystal surface into which molecules are incorporated, building crystal layers in a spiral fashion around the dislocation core. This mechanism is realized basically at lower supersaturations, about 1–1.5. At higher supersaturations, protein crystals grow typically by two-dimensional nuclei formed on the surfaces. Absorption of three-dimensional nuclei have also been detected. The three-dimensional clusters are developed into multilayer stacks or microcrystals.

Absorption of impurities may cause a cessation of growth of macromolecular crystals. Dubrin and Feher revealed parallel step trains on lysozyme crystal surface accounted for by impurities, impeding the subsequent crystal growth. Malkin *et al.* have detected surfaces of lysozyme crystals completely covered by impurity absorption layers resulting in the cessation of growth.

Methods of Crystallization

Proteins may initially contain a large amount of impurities, such as salts, other classes of macromolecules, denaturated molecules, solid particles and other contaminants impeding crystallization. Therefore, proteins should be purified for crystallization tests. The same applies to buffers, salts and precipitants used in crystallizing solutions. Descriptions of preparation and handling of proteins for crystallization and methods of characterization of macromolecules can be found in Further Reading.

There are many methods, techniques and apparatus for protein crystal growth depending on configuration of experiment – hanging drop, sitting drop or crystallization in a capillary. The method used also depends on the way the solution is supersaturated: by vapour diffusion, liquid–liquid diffusion, dialysis, mixing with a precipitant, change of temperature, etc. Other factors include gravity conditions and mass transport in solution, crystal growth in microgravity, gel method, crystallization in drops and suspended in heavy liquids.

Hanging and Sitting Drop

A hanging or sitting drop comprising a solute protein, to be crystallized, with a crystallizing agent, is equilibrated against a reservoir solution containing the crystallizing agent at higher concentration than in the drop (**Figure 1**). The reservoir, a glass vessel, is closed by a glass cover slip and sealed by grease to prevent the liquids evaporating. The drop volume is usually from 1 to 10 μL, the volume of reservoir solution is ~0.5–1 mL. An increase of concentration of crystallizing agent in the drop leads to a supersaturation of protein solution necessary for crystallization. Equilibration is achieved by vapour diffusion of water or other volatile components and continued until vapour pressures in the drop and in the reservoir become equal.

The crystallizing agent may comprise a buffer, such as sodium acetate, tris hydrochloride, HEPES or sodium cacodylate, a precipitant, such as polyethylene glycol, isopropanol or sodium formate, and a salt.

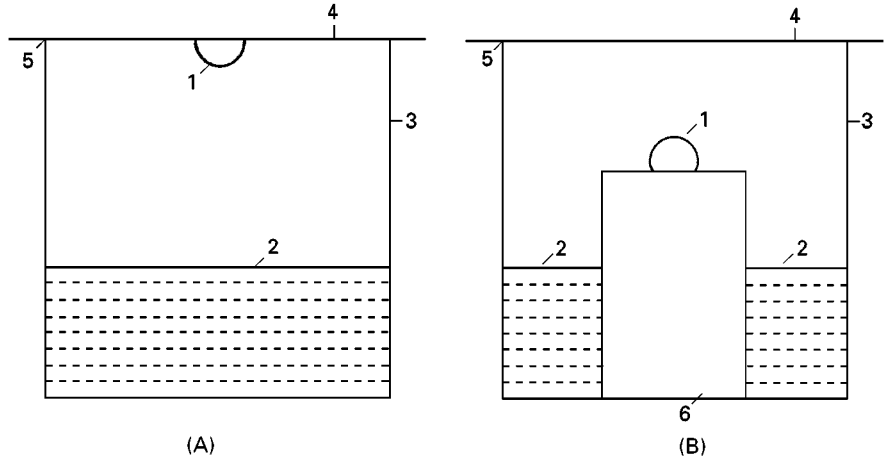

Figure 1 Schematic presentation of hanging- and sitting-drop techniques. (A) Hanging drop; (B) sitting drop. (1) Drop of protein solution, (2) reservoir solution, (3) glass vessel, (4) coverslip, (5) sealing rim, (6) inverted glass pot. (From Ducruix and Giedge (1992) by permission of Oxford University Press.)

If there are no volatile components except water, diffusion of water occurs from the drop to the reservoir solution. This decreases the drop volume and accordingly increases the concentration of all the components. In the presence of a volatile species, such as isopropanol, in the crystallizing agent, diffusion proceeds in two directions, from the reservoir to the drop, and in the opposite way, from the drop to reservoir, of water. Equilibrium is achieved after the saturated partial vapour pressures of evaporating components become equal in the drop and in the reservoir. In this case, the drop may decrease or increase in volume or remain the same.

The often used ratio between concentrations of the crystallizing agent in the reservoir and the drop is 2. This is obtained by mixing equal volumes of protein solution and the reservoir solution. It may be, for instance, a mixture of 2 μL of the reservoir and 2 μL of the protein solution in the hanging or sitting drop. If only water diffusion takes place in the absence of other volatile species, at equilibrium the final volume of the drop will be half the original volume. Accordingly, concentration of all components in the drop, protein, buffer, salt and precipitant double.

Nucleation and crystal growth in a drop are affected essentially by rate of equilibration. The rate depends on the difference between vapour pressures in the drop and reservoir, which in turn depends on the content of the crystallizing agent, temperature, and the size and shape of the drop. Significantly lower equilibration rates with polyethylene glycol rather than ammonium sulfate as a precipitant have been demonstrated. A decrease of equlibrium rate with increase in drop size and with decrease of temperature has also been revealed. The time for $\sim$90% equilibration varies from a day to about one month.

In the hanging-drop technique, crystals are basically created at the bottom of the drop, near the surface. This may be caused by forming a layer of supersaturated protein solution near the surface during evaporation of water. The subsequent distribution of protein concentration over the whole drop is diffusion limited.

The contribution of convection is small, since the supersaturated layer is of higher density and located at the bottom of the drop. Such inhomogeneity applies to nucleation as much as to crystal growth.

In the sitting-drop, distribution of protein concentration is different. Evaporation forms initially a layer of higher concentration near the surface at the top. This excessive concentration is then distributed quickly over the drop by convection. Accordingly, nucleation and the start of crystal growth occur practically at the same protein supersaturation. This is a disadvantage in comparison with the hanging-drop technique, since crystals grow at higher supersaturation. From another point of view, however, the sitting-drop method is energetically more favourable and heterogeneous nucleation on various substrates, including minerals can take place.

For screening and optimization tests, multichamber plates are utilized, containing many wells of the type shown in Figure 1. Plastic Linbro boxes, normally used for tissue culture, are convenient for hanging-drop tests. Each such box contains 24 wells. To prepare tests, about 0.7–1.0 mL of crystallizing agent is put in each reservoir. A drop comprising a mixture of protein solution and reservoir solution is placed on the glass coverslip. Then, the coverslip with the drop is gently turned over and set on the vessel rim covered with a thin layer of grease. In a similar way, the sitting-drop tests are prepared, with the difference that the drop is placed on an inverted glass pot (Figure 1).

Method for Crystal Growth in a Capillary

A vapour diffusion method for growing crystals inside capillary tubes, invented by M. Y. Gamarnik and U. R. Alvarado, is illustrated in **Figure 2**. The crystallizing unit comprises a capillary tube containing a column made up of three layers. The first layer is a protein solution to be crystallized, which may contain a buffer, salt, precipitant and other additives. The second layer is an absorbent, which is typically a liquid, such as glycerol or a highly concentrated salt solution. In the preparation of the crystallizing unit, the protein solution and absorbent are placed in the capillary tube so that they are segmented by an air section. The ends of the capillary tube are sealed by end-caps. The liquids are placed sequentially in the capillary tube by a syringe. The lengths of the protein solution, absorbent and air layer segments are selected by experimenter, but the usual lengths are from 2 to 20 mm.

The internal diameter of the capillary tube is selected so that the capillary forces, acting on the liquids, held within the sealed tube, are sufficient to prevent direct contact between the liquids during handling or transportation. The internal diameter is usually less than 3 mm, but for crystallization tests from about 1–10 μL of protein solution the diameter should be about 0.6–1.5 mm.

The vapour of the solvent, which is normally water, diffuses from the protein solution through the air layer to the absorbent because of the difference in the water vapour pressures in the protein solution and absorbent. Evaporation of water from crystallizing solution results in an increase of concentration of protein and other components – buffer, salt and

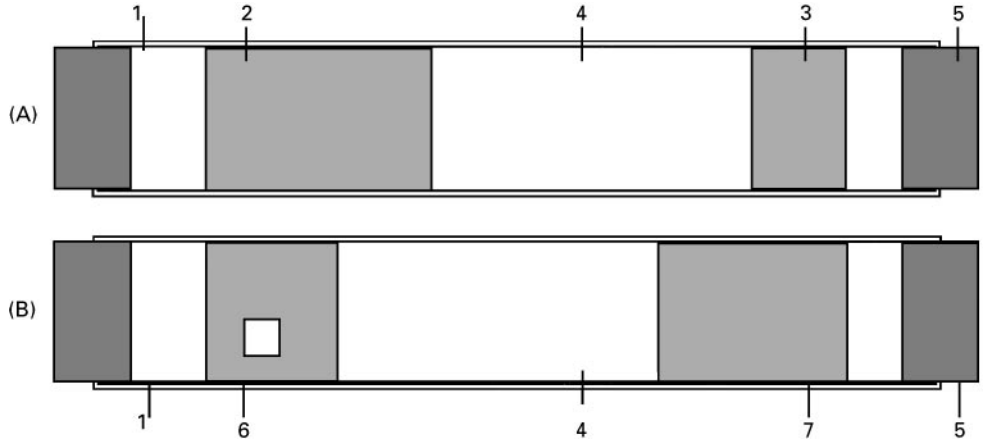

Figure 2 Schematic presentation of vapour diffusion method for crystal growth inside a capillary tube. (A) Initial configuration, (B) final configuration. (1) Capillary tube, (2) protein solution, (3) absorbent, (4) air layer, (5) end-caps, (6) protein solution with grown crystal, (7) absorbent containing absorbed water.

precipitant – which were initially present. Nucleation and growth of a protein crystal occur within the crystallizing solution after it reaches the appropriate supersaturation.

During the vapour transport, the length of the air layer remains approximately constant but shifts toward the crystallizing solution. The length of the protein solution layer decreases. Accordingly, the length of the absorbent, containing absorbed water, increases (Figure 2B). At any time during crystal growth, it is possible to calculate the concentration of protein and other additives in the protein solution by the relative change of its length.

A desired rate profile of water evaporation can be set by appropriate choice of concentration and volume of the protein solution and absorbent, the length of the air layer and the temperature. Often it is desirable to set a relatively rapid rate of evaporation during the initial phase of the growth process leading to nucleation, followed by a lower rate of evaporation during the crystal growth process. To accomplish this profile, the air layer length should be small, about 3–5 mm, and the absorbent layer length should be also small, in comparison with the length of protein solution layer. The short air layer establishes a relatively fast dewatering followed by a rapid increase of concentration of protein solution, while the small volume of absorbent allows a relatively rapid decrease in the rate of evaporation after the initial phase of evaporation, due to dilution of the absorbent by the absorbed water. This profile, initiating nucleation with subsequent slow crystal growth, usually yields a single crystal or a few crystals in each portion of the protein solution. This is beneficial since the dissolved protein feeds only one or a few crystals during the growth, resulting in their larger size.

Liquid–Liquid Diffusion Techniques

Interface diffusion In the interface-diffusion method for protein crystal growth two liquids – a protein solution and a solution of the crystallizing agent – make contact and are separated at an interface. Equilibration is achieved by diffusion of the crystallizing agent and protein across the liquid–liquid interface. Diffusion leads to a slow increase in the concentration of the crystallizing agent in the protein solution near the interface. Nucleation occurs in the interface region after sufficient supersaturation. Subsequent slow crystal growth is provided by the relatively small interface region of the higher protein supersaturation.

A basic difficulty in this technique is bringing the liquids into contact without convection, i.e. without rapid mixing. Convection may be reduced if the less dense solution is gently placed on the more dense solution. Crystallization experiments conducted under microgravity conditions in space avoided convection during solution contact when the interface-diffusion technique was used.

To use only a small amount of protein solution, about 2–20 μL, the liquids may be brought into contact in a capillary tube of diameter in the range $\sim$1–2 mm.

Dialysis In the dialysis technique, the protein solution and crystallizing-agent solution are separated by means of a molecular membrane. Both solutions are in contact with the membrane, which allows diffusion of small molecules, but prevents macromolecules passing. The maximum molecular weight of molecules able to diffuse through the membrane pores is determined by the molecular weight cutoff (MWCO).

The MWCO of contemporary dialysis membranes is in the range from 100 to 300 000 Da.

During equilibration, molecules of buffer, salt, precipitant or water may diffuse through the membrane from the crystallizing agent to the protein solution and also in the opposite direction, resulting in crystallization. For micro-quantities of protein solution, capillaries of small internal diameters, about 1–2 mm are used. The protein solution is placed in a capillary, the end of which is capped by the membrane. The membrane end of the capillary is then put into an appropriate agent solution for crystallization.

Batch technique Microbatch techniques include crystallization under oil, with droplets of about 1–10 μL of crystallizing solution. It was demonstrated that application of paraffin and silicone oils affect the rate of equilibrium, resulting in a better quality of crystals. A containerless method has been developed, where the crystallizing solution drop is suspended between two oil layers of different density, preventing the undesirable contact of the protein solution with walls of a vessel.

A drawback of the batch method is that, typically, many nuclei are formed resulting in the growth of many crystals, and it takes much effort to find the conditions for growing just one or a few crystals in a single batch.

Gel Method

In the gel method, crystallization in a gel results in the protein solution being trapped by a loose network which is stretched over the whole volume of the solution. The gel comprises macropores, of about 100 nm in size, filled with the crystallizing solution. The macropores are interconnected, by a dense system of micropores, of about 10 nm in size.

Entrapping of the crystallizing solution by the gel network prevents natural convection and sedimentation. Accordingly, equilibration between the protein solution and the crystallizing agent is mediated by diffusion only, through the gel pores. This results in slow crystal growth, improving the quality of the produced crystals if no gel structure is incorporated in the crystal lattice, as is often the case. Various crystal growth techniques can be used with a gel, such as hanging-drop technique, liquid–liquid diffusion and dialysis. Silica gel and agarose gel are most often used for protein crystallization.

Crystallization in Microgravity

Protein crystal growth has been studied under microgravity conditions conducted in space (satellites and space shuttles). Space-grown crystals are frequently of better quality than the same protein crystals grown on the earth. Under microgravity conditions, convection and sedimentation are suppressed. Accordingly, transport of molecules, supersaturating the protein solution, is mediated by diffusion only, providing slow crystal growth. Typically, crystals are suspended and grow freely in different crystallographic directions, forming a well-ordered structure and equilibrium shape.

Various methods have been used for crystal growth experiments in space, including interface-diffusion, dialysis and vapour diffusion. Most methods have a device or a means to connect or disconnect interaction between the protein solution and crystallizing agent so that mixing only takes place under zero gravity. Typically, two wells, one of which is filled with protein solution and a second one filled with crystallizing agent, are brought into contact in orbit through a connecting valve or by a relative movement of the wells.

Concluding Remarks

Experiments, directed at crystallization of proteins require an understanding of the main principles of nucleation and growth and need much testing. Some tests are associated with a decrease in critical supersaturation, necessary for nucleation, through selection of appropriate buffers, salts and precipitants or by exploration of substrates for heterogeneous nucleation. Other methods follow the development and use of crystallization cells, consuming small amounts of protein solution, through decreasing the internal diameter of capillaries (in the capillary method) or the size of droplets in microbatch and hanging-drop experiments.

Small volume crystallization cells will be adapted for microgravity experiments to reduce space requirements on satellites and shuttles, accordingly reducing their cost. This may be realized by modification of the capillary technique, shown in Figure 2. For instance, two air layers may be used instead of one, separated from each other by a water barrier layer. The water barrier layer delays absorption of the vapour from the protein solution until the barrier layer is eliminated by absorption into the absorbent. The necessary delay is typically one or two days, from preparation of the tests until the spacecraft carrying the crystal growth device has attained microgravity conditions.

Development of crystallization methods at lower critical supersaturation seems to be supported by a broadening of our knowledge of the main principles governing nucleation and growth of macromolecular crystals.

See also: **II/Crystallization:** Additives: Molecular Design; Control of Crystallizers and Dynamic Behaviour; Polymorphism. **III/Supercritical Fluid Crystallization.**

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Electrophoresis

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Transmissible spongiform encephalopathies are neurodegenerative diseases found in both humans and animals. The oldest known member of this family of diseases is scrapie in sheep and goats and the most infamous member is bovine spongiform encephalopathy or 'mad cow disease'. These diseases are fatal for the individuals who become infected. As a result, there is a considerable amount of interest in developing methods to detect early infection. This would enable removal of animals from food chains and by-products used for cosmetic and health care. For humans, an early diagnosis may make it possible to treat infected individuals with drugs to arrest the course of the disease.

A feature of these diseases is the accumulation of rod-shaped fibrils in the brain that form from an aggregated protein. This abnormal protein is a protease-resistant form of a normal host cell glycoprotein (prion protein). When the aggregated protein is denatured in sodium dodecyl sulphate (SDS) and β-mercaptoethanol, a monomer form of $M_r \sim 27$ kDa is observed. This abnormal prion protein is used as a marker for infection with a transmissible spongiform encephalopathy.

The abnormal prion protein is insoluble in most biological buffers, whereas the normal prion protein is soluble. In natural infections, the abnormal prion protein is found in very low concentrations. It is found in higher amounts in rodent-adapted strains of the disease. These properties of insolubility and low concentrations present quite a challenge for the development of analytical methods to detect this protein.

Most of the methods used to detect the prion protein are based on histological techniques and are used postmortem. Immunoassays can be used to measure the prion protein. Most of the antibodies that have been produced to the abnormal prion protein have been made to the denatured form. Removing these denaturants is a major problem in the development of immunoassays. Western blot can be used to detect the prion protein but cannot be easily automated. Some new approaches using chemiluminescence and time-resolved fluorescence in plate assays have been developed. The amount of prion protein detected by these assays is in the range of picomoles or > 500 fmol. To improve sensitivity, we have approached this problem using capillary electrophoresis with laser-induced fluorescence. Fluorescent-labelled peptides from immunogenic epitopes of the prion protein can be detected in the attomole range using this technique. A competition immunoassay using fluorescein-labelled peptides was developed which is