Chapter 2
Basic Concepts of Density Gradient Ultracentrifugation

Jindi Wang, Jun Ma and Xuemei Wen

Abstract This chapter reviews the development of centrifugal equipment and its achievements in various fields, especially in the field of life sciences. Based on the development of the equipment, centrifugal technology also plays a very crucial role in scientific research. Afterward, we introduce three common types of separation techniques: differential centrifugation, rate-zonal centrifugation, and isopycnic separation. For each type, we describe the basic principles, characteristics, and scope of application in detail.

Keywords Density gradient ultracentrifugation · Revolution of centrifugation Differential separation · Isopycnic separation · Rate-zonal separation

2.1 Revolution of Density Gradient Ultracentrifugation

Centrifugal technology has been developed for centuries. As early as the ancient times, people utilized centrifugal force by using tied rope to circularly move pot to squeeze honey or slurry. The dehydration and separation processes from textile and light industry in mid-eighteenth century facilitated the appearance of modern model of the centrifuge. In 1836, the first three-foot centrifuge came out in Germany, and a cotton dehydrator also appeared. In 1878, the Swedish de Laval developed a manual-belt-type milk separator and used it to make cream. Six years later, he invented the butter separator driven by the diesel engine to further improve the separation efficiency. These types of equipment greatly promoted the development of the cream industry. During this period, with the rapid development of industry, centrifugal technology has also made great progress. However, due to the limitations of science and technology at that time, high-speed centrifugal equipment could not be realized, which greatly hindered the application of separation technology in the field of basic science.

Since the twentieth century, the industrial manufacturing techniques have been greatly improved. High-speed centrifuges and ultrahigh-speed centrifuges were produced one after another. In the 1920s, US company "DuPont" produced the oil turbine centrifuge. In 1933, the air turbo centrifuge was developed by using the

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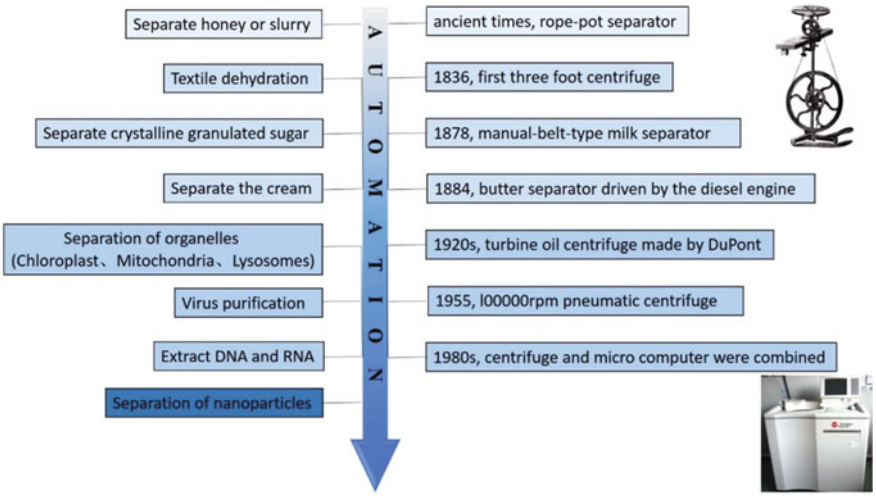

Fig. 2.1 Shows the important development of centrifugation

compressed air to drive the worm wheel and then drove the centrifuge to rotate. In 1955, US company "Beckman" launched up to l00,000 rpm pneumatic centrifuge; in the late 1970s, variable frequency motors emerged. Then, in the 1980s, micro-computers were integrated with the variable frequency motors, so the speed and performance of the centrifuge were highly improved. The continuous improvement in centrifuge extends and strengthens the exploration in many scientific fields, especially in the life sciences. As shown in Fig. 2.1, we have summarized some representative developments of centrifugation.

As the research of cells gradually goes in-depth, the study of subcellular structures has become a hot topic in life sciences, which demands the pure organelles from cell. Hence, scientists began to use the centrifugal method to obtain organelles from the cell brei. The first breakthrough was made in 1934, and Bensley and Hoerr [1] used high-speed centrifugation to isolate mitochondria from the cell homogenate. Consequently, they studied the chemical composition and physiological function of mitochondria and concluded that mitochondria are the center of cell oxidation. In that case, biology had ushered in a new development period.

Extending from prior successes, Hogeboom et al. [2] put forward a complete separation protocol describing how to separate various suborgans from the cell homogenate in detail. This protocol is also represented as the preliminary formation of differential centrifugation model.

The differential centrifugation method, also known as pelleting separation, which is a method of using a certain speed centrifugation to separate materials from homogeneous suspension, has been widely applied to separate and purify nanoparticles by centrifuge redispose circles. In the 1950s, Brakke set up a rate-zonal separations method. He proposed the concept of density gradient

centrifugation for the first time [3] and demonstrated this new method for virus purification. On the basis of this method, Meselson et al. [4] proposed a balanced gradient centrifugation method, with the gradient distribution of DNA in CsCl. De Duve's group [5] used density gradient centrifugation method for the first time to get the lysosome. This work represented another major leap in human understanding of the cell; Duve also won the 1974 Nobel Prize in physiology or medicine. This series of meaningful works made the density gradient centrifugation attract more and more scientific attentions. After that, the centrifuge technology had been greatly improved.

Now, density gradient centrifugation technique is widely used to study the biological characteristics and the separation of biological samples on cells, subcellular organelles, nucleic acids, proteins, enzymes, and receptors. It has become one of indispensable technical means in life sciences. With the rapid development, density gradient centrifugation technique not only plays an increasingly great role in biological field, but also promotes the continuous technology improvement and development in broader application prospects.

2.2 Differential Centrifugation

2.2.1 Introduction

Differential centrifugation is a centrifugal method based on the different settling velocity of nanoparticles in a homogeneous medium. As shown in Fig. 2.2, the technique is accomplished by repeatedly centrifuging a suspension from low speed to high speed to achieve separation. In a typical process, different sized particles move toward the bottom of the centrifuge tube with different settling rates and eventually precipitate. Specifically, in order to separate the different components, we can adjust the rotation speed to change the applied centrifugal force and further to manipulate the settling velocity of the particles. This centrifugation is also called differential pelleting, because the samples can be separated by sequentially increasing the centrifugal speed to obtain a series of precipitations and supernatants.

Next, we give a brief description of how to select the centrifugal speed. As shown in Fig. 2.2, firstly, low speed can be used to make the largest particles settle at the bottom of the centrifuge tube, which are precipitation 1. After removing the precipitate 1 and resuspension, a medium speed should be used to obtain the medium particles. Finally, the precipitation of the smallest sized particles needs the highest centrifugation speed. Through the above steps, the preliminary separation of different components can be realized. In particular, it can be seen from the tubes that the fractions obtained by this approach are not strictly monodispersed.

The applications of differential centrifugation are widespread, especially in biochemistry for the separation, extraction, and enrichment of bioactive substances, such as animal/plant virus and subcellular components. Figure 2.3 shows a typical

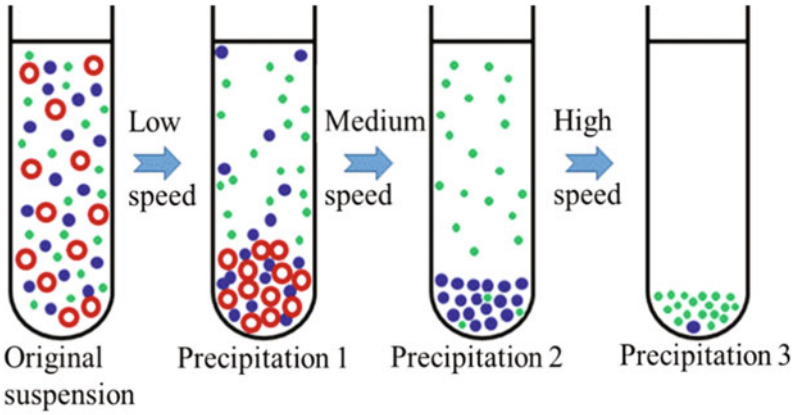

Fig. 2.2 Basic process of differential centrifugation

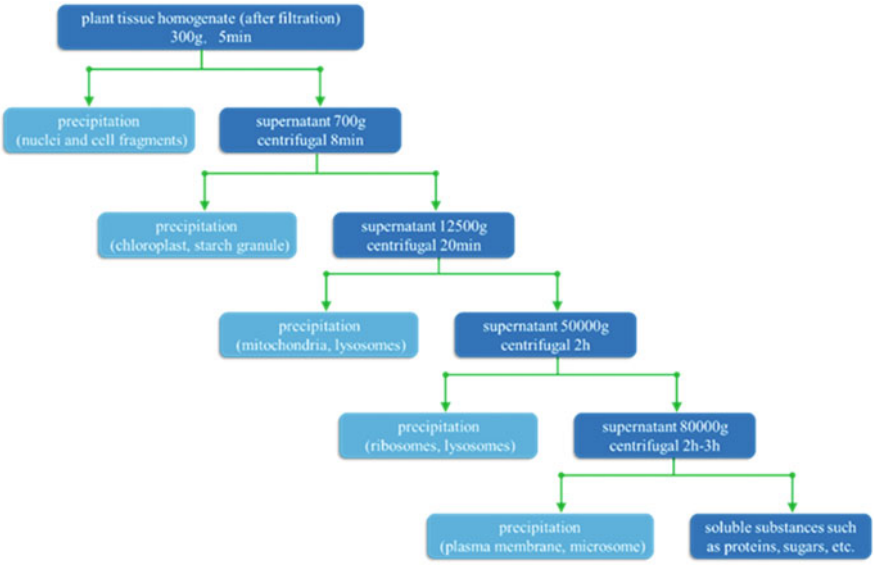

Fig. 2.3 Differential separation process of subcellular structures of plant materials [3]

differential separation process of subcellular structures of plant materials. Samples got from the precipitation must go through the resuspension, recentrifugal, and rewashing processes to get much purer samples of the particles. The yield by differential centrifugation is not high. Except for the slowest settled particles in the group, however, it is hard to get a pure component.

2.2.2 Basic Principle

When the particles are settled in centrifugal field, their sedimentation speed depends on many parameters, such as mass, size, shape of particles, and density, viscosity of media. The detailed information will be discussed in Chap. 4. Here, we will give basic principles of differential separation.

For a particle (suspended in a liquid of known density and viscosity) of a given size and density, the density and viscosity of the liquid medium are known quantitatively, and for a certain particle, r, ρ_p, η, and f are also known quantitatively, and the sedimentation coefficient (s) can be calculated as $s = \frac{2r^2(\rho_p - \rho_m)}{9\eta(f/f_0)}$ and

$$\frac{dx}{dt} = s\omega^2 x$$

For convenience, we mark the sedimentation coefficient as follows

$$s = \frac{dx/dt}{\omega^2 x}$$

Accordingly, the sedimentation coefficient is the ratio of particle velocity (dx/dt) to its acceleration ($\omega^2 x$), which determines the sedimentation rate of particles, and is of great importance for the sedimentation behavior of particles in differential separation.

2.2.3 Applicable Conditions

In a typical process of differential separation, all the different sized particles move toward the bottom of the centrifuge tube. As mentioned above, the differential separation is based on the different settling velocity of nanoparticles in a homogeneous medium and determined by the sedimentation coefficient.

For an intuitionistic comparison, three types of particles with 100S, 10S, and 1S (S = $10^{-13}s$, s is sedimentation coefficient) are mixed and sorted by differential separation, and the separation results are quantitatively analyzed, as shown in Fig. 2.4.

It is obvious that, after the optimization of differential separation parameters, there are still 1% of smallest sized particles and 10% medium-sized particles precipitated with largest particles. When applying the precipitation process again, there are still some big-sized precipitates mixed in small-sized precipitates [7]. Washing precipitation can improve the purity of separation, and this process can resuspend and centrifuge the precipitation to improve the efficiency of differential

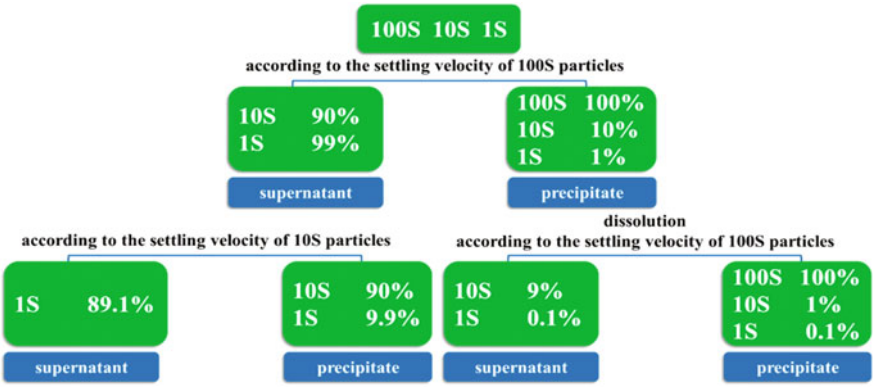

Fig. 2.4 Separation result of the particles of three sedimentation coefficients in the sample: 100S, 10S, and 1S

centrifugation. By using this method, the maximum-sized precipitation with high purity can be obtained after several washing.

Thus, the differential separation method is suitable for the samples which contain big weight difference between the fragments, or the difference between sedimentation coefficients (the measurement on settlement, which is defined as the quotient of a particle's sedimentation velocity over the acceleration) of mixed samples should be more than ten times.

2.2.4 Basic Calculation

Angular rotor is often used in differential centrifugation method, and the particles are deposited on the outside wall of centrifuge tube as moving toward the bottom (Fig. 2.5) [8].

According to the definition of sedimentation coefficient:

$$s = \frac{dr/dt}{w^2 r}$$

we can get:

$$s \int_{t_1}^{t_2} w^2 dt = \ln \frac{r_2}{r_1}$$

Centrifugal time is the centrifuge to run from start to run completely stop, and the expression is:

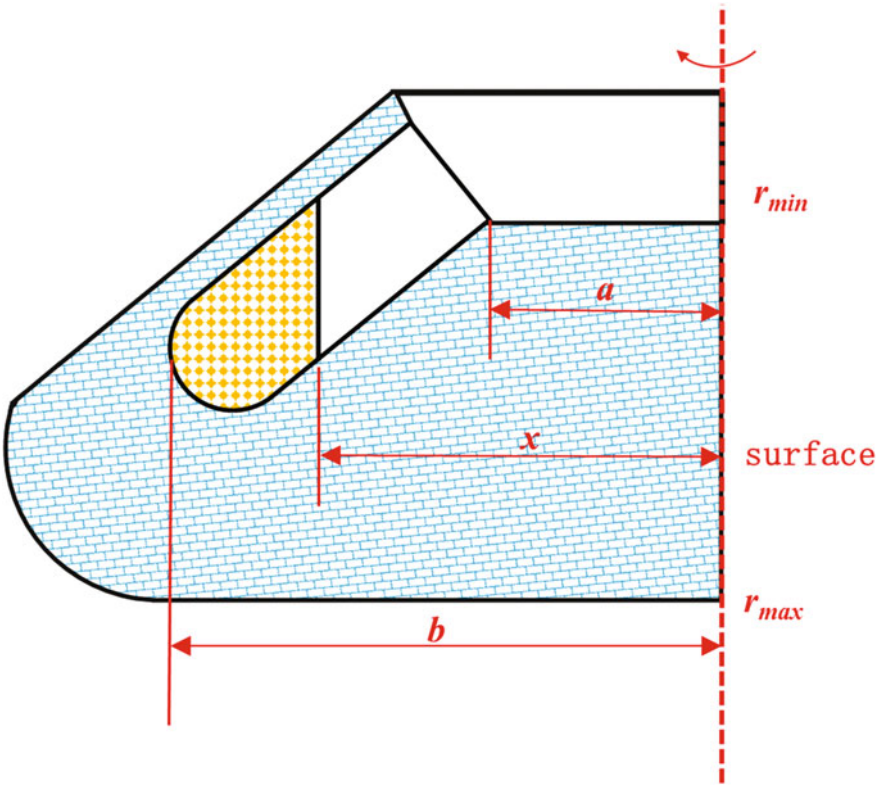

Fig. 2.5 Movement of the interface in the angular rotor. *a* Minimum centrifugal radius; *b* maximum centrifugal radius; *x* the distance of the particle mobile interface

$$\int_{t_0}^{t_{\text{stop}}} w^2 dt = \int_{t_0}^{t_{\max}} w^2 dt + \int_{t_{\max}}^{t_{\text{off}}} w^2 dt + \int_{t_{\text{off}}}^{t_{\text{stop}}} w^2 dt$$

The time of the acceleration and deceleration is different among kinds of centrifuge. The actual effective expression is as follows:

$$t = \frac{1}{3}(t_{\max} - t_0) + (t_{\text{off}} - t_{\max}) + \frac{1}{3}(t_{\text{stop}} - t_{\text{off}})$$

Or

$$t = \frac{1}{3}t_+ + t_{\text{constant}} + \frac{1}{3}t_-$$

If *s* value of the particle is known, the whole matter of the size will be settled down (Fig. 2.6).

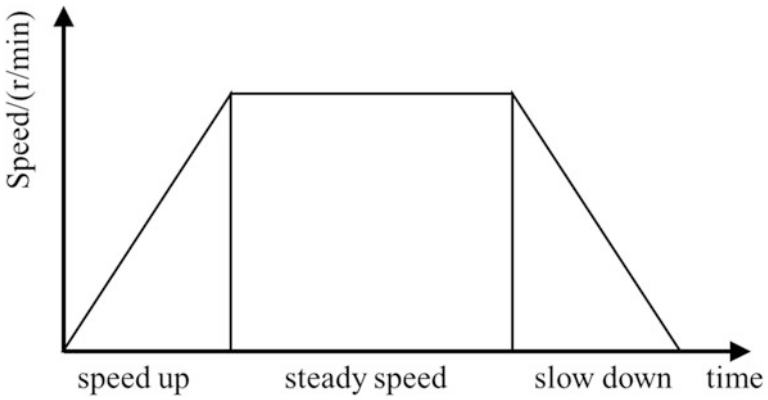

Fig. 2.6 Scheme of actual effective centrifugal time calculation

$$b = ae^{sw^2t}$$

$$\ln\frac{b}{a} = sw^2t$$

Known

$$w = 2\pi n$$

For any r and t, the following two equations are used:

$$n^2t = \frac{3600}{4\pi^2 s}\ln\frac{b}{a} \times \frac{1}{n^2}(s)$$

$$n = \sqrt{\frac{3600}{4\pi^2 s}\ln\frac{b}{a} \times \frac{1}{t}}$$

2.3 Rate-Zonal Separation

2.3.1 Introduction

Rate-zonal separation is a kind of centrifugation method using zone rotor [9], which is based on the differences in sedimentation velocities of samples in density gradient [10]. The method is to put the samples on the top of the density gradient which has a smaller scale of density ($\rho_{\text{media}} < \rho_{\text{particle}}$) and mildly changed slope. Mass and the viscous resistance are different for the particles with different shapes and sizes under a given centrifugal force, which leads to different sedimentation coefficients and consequent different particle sedimentation velocities, and effective separation.

Typical zone rotor is a hollow pot body, including upper and lower parts, which is convenient to clean and maintain. It cuts down the amount of centrifuge tubes and greatly increases the use of volume. The center of rotor is axis and partition, and the rotor cavity is divided into several regions by partition, which can reduce the eddy current and stabilize gradient liquid. Zone rotors are divided into redirect, non-directional, continuous flow, etc. Redirect rotor is that gradient medium and samples in the rotor present radial in the process of centrifugal acceleration, and slow down the process of orientation change according to the gravity, gather gradient zones from the top or the bottom after centrifugal separation. Continuous flow zone rotor is that samples or medium are added through the rotation of coaxial rotor rotating sealing device when the machine is running. After the completion of the first centrifugation, centrifugation is further running through the same rotating sealing device of continuous pumping medium to replace the separation zone. Also, the isolated samples are collected through monitor testing part.

Usually, by using zonal rotors and swing-out rotors, and taking advantage of their long settlement path, the particles can be fully separated. Sucrose and iodinated gradient media are mostly used. The centrifugation process should be stopped when the fastest settling particles reach the bottom of the rotor or wall of tube. Rate-zonal separation undertakes in strong centrifugal field and costs short period of separation time (generally not more than 4 h). The sample particles would go through the gradient and stack at the bottom of the rotor if the centrifugal time is too long. In order to avoid such situation, a cushion layer whose density is greater than the maximum grain density is required to be put at the bottom of the gradient.

In homogeneous medium, the interactions between the particles and medium should be the same in the process of settlement. Under a given centrifugal field, the settling velocity of particles increases with the increase of the centrifugal radius. In that case, the centrifugal radius of leading particles would increase faster than that of slow ones, and consequently, settling velocity of leading particles would also increase faster, leading to the enhanced resolution. While in the density gradient, density and viscosity of medium continuously increase, and leading particles would enter into the higher density area first. Thus, sedimentation velocity of leading particles will slow down quickly due to the strong resistance. The greater the slope of gradient, the greater the speed goes down, which will be harmful to improve separation resolution. However, if there is no density gradient, all particles would be settled at the end of centrifuge tubes. At the same time, the convection produced by the change of the rotor speed can mix separated particles again in homogeneous medium [11]. Hence, in order to obtain maximum separation resolution, the samples suspension added on the gradient should be as thin as possible, viscous resistance and buoyancy of particles in a given centrifugal field by gradient moving should be minimal, and the separation should be carried out as soon as possible after the formation of density gradient to minimize the diffusion effect (Fig. 2.7).

To guarantee the stability of sample zone, the sample weight added in the density gradient liquid column should be matched with the concentration of the

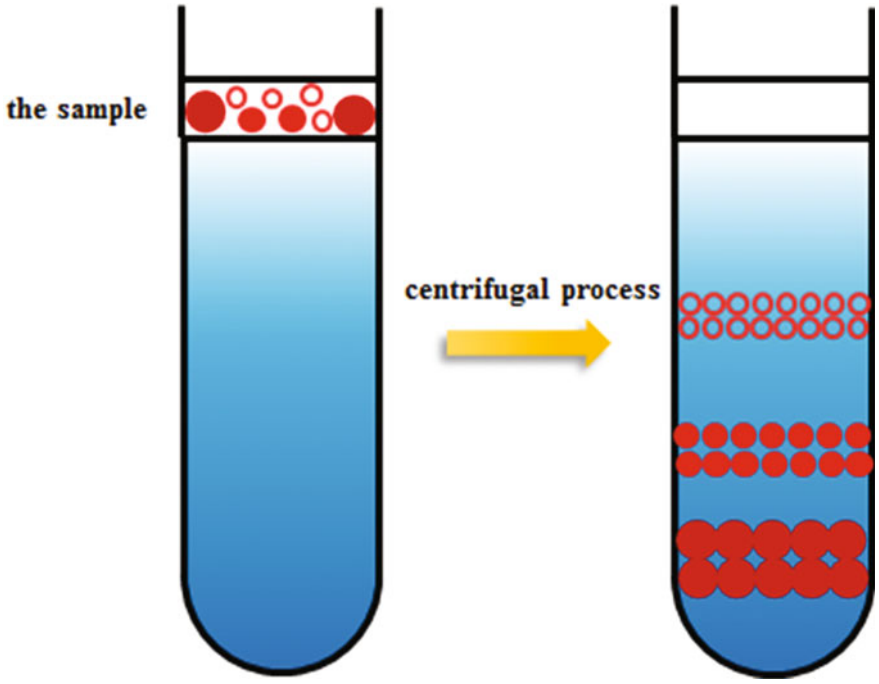

Fig. 2.7 Schematic of rate-zonal separation

sample. The maximum density of gradient (usually the bottom gradient) must be lower than that of particles, and the minimum density of gradient (usually the top gradient) must be greater than the density of sample suspension. If the subsidence coefficient of different particles is similar, gradient density with smaller range should be chosen, and if the differences of particle density are large, the steep gradient will be preferred [12]. If the sample concentration is too high, the zone will be severely spread; while if the sample concentration is extremely low, it would be hard to identify the sample zone [13].

2.3.2 Basic Principle

The particle's settling velocity in the centrifugal field is influenced by the density difference between the particle and surrounding medium, particle size (e.g., diameter and length), centrifugal field force, and viscous drag (associated with the viscosity and surface area of the particle). The calculation can be expressed by the following equation

$$v = kgr^2 \frac{d_1 - d_2}{\mu}$$

v Particle settling rate (cm/s).
g Gravity acceleration.
r The radius of the particle (cm).
d_1 The specific gravity of particles.
d_2 Specific gravity of water medium.
μ Viscosity of water medium.
k The shape factor varies with shape

The speed of any particle (dr/dt) goes up with the increase of the rotor radial distance r (i.e., increases with r). It is obvious that the dr/dt in the density gradient is not depended on r, and it changes with the density and viscosity of the medium. In the later discussion of the method, the changing in the settlement velocity with the density of the medium will be unveiled, and the estimated settling time under different conditions will be demonstrated. The basic principle of particle subsidence is the same as the differential velocity centrifugation.

2.3.3 Considerations

All in all, there are some guidelines that need to be followed in rate-zonal centrifugation. Firstly, the density of particle suspension must be lower than that of the top gradient. Then, the density of the sample particles must be higher than that of bottom gradient. In addition, the path length of the gradient must be long enough to sufficiently allow the particles to be separated. Finally, the centrifuge should be stopped before or as long as the leading particles reach the bottom of the gradient.

2.3.4 Applicable Conditions: High Density Nanostructures

Rate-zonal separation is a dynamic method related to particle size and density. Buoyancy and the viscous resistance are different for the particles with various shapes and the size in the density gradient, which leads to different sedimentation rates, so under the condition of a certain centrifugal, particle size, morphology, and structure can be effectively separated. Figure 2.6 shows process of rate-zonal separation. Compared with isopycnic separation, rate-zonal separation can be used for separating these particles, density of which is much higher than the gradient medium of colloidal particles, such as metal nanoparticles, which greatly expands

the scope of separation. And the time of rate-zonal separation (such as 15 min) is shorter than typical isopycnic separation time (usually 12 h). In addition, parameters for the separation, such as time, centrifugal velocity, and gradient density, can be adjusted specifically for the separated particles.

The rate-zonal separation method has been successfully applied to sort the various colloidal nanostructures, such as FeCo@C, Au nanoparticles, graphene, and CdS nanorods [14].

2.4 Isopycnic Separation

2.4.1 Introduction

When the density of particles suspended in the mixture suspension is different from that of the gradient medium, under the effect of the centrifugal force, they either settle or rise along with the gradient. The particles will keep moving until they reach the same density of gradient. At this point, the particles are in the minimized energy state [15]. Here, the gradient medium density is equal to the buoyancy density of particles, which is considered as "isopycnic state." This centrifugal method, which establishes and measures the equilibrium state, is considered as isopycnic equilibrium gradient centrifugation. The effect of the density isopycnic centrifugal separation depends on the density difference between the particles and separation ability of gradient medium. The particles with bigger density difference are easier to separate since the separation effect is regardless of the size and shape of the particles for isopycnic one. However, the size and shape have a profound effect on the equilibrium time and the width of the zone. In isopycnic equilibrium gradient centrifugation, the buoyancy density of particle is not constant and highly related to the density of the particle and hydration degree, and it also relates to these factors such as gradient media and interactions. For example, some particles are easy to be hydrated, so their net density will be lowered; sometimes the net density would be even changed due to the replacement of surface structural water by small molecules of gradient medium. The influences of these factors should be taken into consideration when colloidal nanoparticles are separated.

In practical, sample should be loaded on preformed gradient with increasing density before centrifuge starting; after long-term centrifugation (usually longer than 12 h), the particles would find the layer with same density to stop and locate therein (Fig. 2.8).

The conditions of isopycnic equilibrium gradient centrifugation depend on the requirements, properties, medium, gradient characteristics of experiment [16].

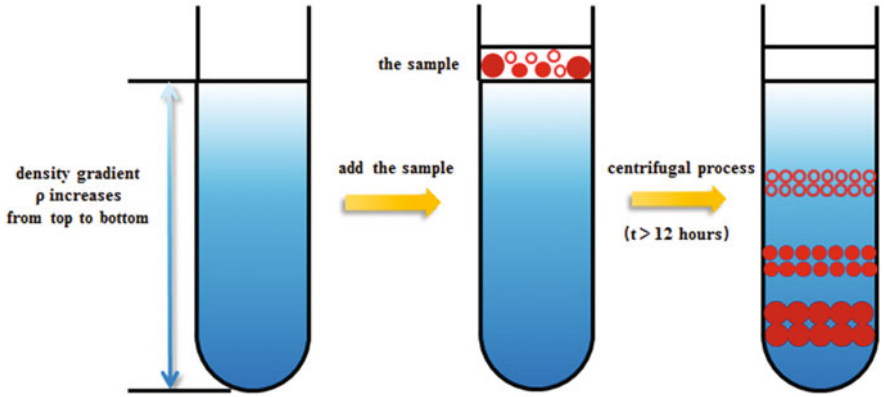

Fig. 2.8 Schematic of isopycnic separation

2.4.2 Basic Principle

For the physical balance in centrifugal field, concentration distribution can be calculated according to the following formula:

$$\frac{dc_m}{dr} \times \frac{1}{c_m} = \frac{\omega^2 r_\theta M(1 - \bar{\upsilon}\rho s)}{RT}$$

Assuming that the relationship of the density and gradient concentration distribution is:

$$\rho_s(r) = \frac{1}{\bar{\upsilon}} + (r - r_\theta) \times \frac{d\rho_s}{dr}$$

when density balance particle concentration distribution relationship is:

$$\ln \frac{c_\theta}{c_r} = \frac{\omega^2 r_\theta M \bar{\upsilon}(d\rho_s/dr)(r - r_\theta)}{2RT}$$

r_θ Radius of zonal density, corresponding to the particle equilibrium average density

c_r The concentration of the radius

A linear relationship between $\ln c_r$ and $(r - r_\theta)^2$, intercept is $\ln c_\theta$, slope is $\omega^2 r_\theta M \bar{\upsilon}(d\rho s/dr)/(2RT)$.

Conditions of isopycnic equilibrium gradient centrifugation are confirmed according to the requirement of the experiment, sample characteristics, the characteristics of the medium and gradient.

Aqueous soluble gradients are used in isopycnic equilibrium gradient centrifugation, and pH value depends on the nature of the biological sample. In most of the cases, sucrose, glucose, glycerol, sorbitol, tartaric acid, potassium bromide, cesium chloride, potassium chloride, trifluoroacetic acid, cesium, and dextran can be chosen as gradient medium.

According to the density difference between particles, the particles will not move to form a layer upon layer of particles when reaching their respective density regions in gradient separation. The equal density gradient includes the preforming gradient and the self-forming gradient. The self-forming gradient is that the gradient medium mixed with the samples together, sample particle distribution in different density position after centrifugation. The preforming gradient is a way that the gradient liquid is placed in a centrifuge tube according to the differences between light and weight. It includes linear gradient and step gradient. The former is prepared by the gradient instrument or by step gradient, which is formed by diffusion, and the latter is formed by manual placement.

If the maximum density of the gradient medium is higher than the maximum density of the sample particles, or the sample density is between the maximum and the minimum of the gradient density, the sample cannot reach to the bottom of the centrifuge tube. The density gradient range includes the density required to separate the particles. When the particles are separated in the process of centrifugation and settle to a position with equal density, the zone is integrated. In this integrated zone, the density of the gradient liquid is as same as the density of the sample. The effect of such density gradient centrifugation is determined by the density differences of the sample particles. The greater the difference in sample density is, the better the separation effect would achieve [17].

2.4.3 Characteristics

The characteristics of isopycnic separation:

- Its resolution is high and can separate two particles whose density differences are less than 0.01 $\sim$ 0.02 mg/cm^3 (1/10^5 density of water).
- It can resist the disturbance caused by temperature change and acceleration and deceleration. Its anti-convection and anti-disturbance ability is excellent.
- It can handle a large amount of sample simultaneously.
- The sample is suspended in a gradient medium and does not form precipitation, which is beneficial for maintaining biological activity.
- The resolution is related to the profile of terminal density gradient after centrifugation, which is mainly affected by the medium types and centrifuge speed. Extending the time of acceleration and deceleration would help to preserve the separation accuracy.

2.4.4 Applicable Conditions: Low-Density Nanostructures

Isopycnic separation is a method of using density gradient or over-speed centrifugal which depends on the density of the separated material. From the separation of biological macromolecular, to the separation of carbon nanotubes, isopycnic separation has achieved great success. Particles move to the positions where the medium density matches with the density of each colloidal particle under given centrifugal field to separate. There is no relationship between separation effect and the size of the particles; however, separation effect mainly depends on the density differences between particles and gradient medium [18].

Density gradient centrifugation appears to be the most effective in separating nanotubes of smaller diameters, such as carbon nanotubes. Mark C. Hersam's group reported that single-walled carbon nanotubes can be separated in the density range of 1.11–1.17 g cm^3 [19]. Graphene nanosheets with surfactant wrapping are separated, and their net densities are 1.1 g/cm^3 [20]. Isopycnic density gradient centrifugation method reaches a limitation when it is extended to the separation of metal nanoparticles. Such a method requires that the components for separation have densities within a gradient range. Aqueous density gradient media usually have densities less than 1.4 g/cm^3, which is much lower than the density of metal nanoparticles. Therefore, this method is only suitable for the separation of colloidal particles with low density [21]. In order to expand the scope of separating particles, other density gradient centrifugation methods are required.

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