

Chapter 3

Density Gradient Ultracentrifugation Technique



Qian Zhang and Xiong Sun

Abstract As a general, non-destructive, and scalable separation method, DGUC has recently been demonstrated as an efficient way of sorting colloidal nanoparticles according to their differences in chemical, structural, size, or morphology. After the introduction of basic concepts of density gradient ultracentrifugation, for the practical applications, there are various parameters to be considered. Nanoparticles will have different movement ways in different separation systems. In principle, particle movement characteristic in liquid media not only depends on the centrifugal force but also relies on the density, size, and shape of particle and the density and viscosity of the liquid medium and so on, while the gravity and intermolecular force can be ignored. In this chapter, typical parameters such as choice of gradient media, density gradient, rotor type, centrifugation speed, and time will be discussed.

Keywords Gradient media · Step gradient · Continuous gradient
Rotor type · Centrifugal force · ω^2t calculation method
 K' coefficient estimation method

3.1 Choice of Gradient Media

Up to now, only a few types of liquids or solutions are utilized to form density gradients to separate biomolecules or nanoparticles (NPs). Since NPs are not biologically active, harsher conditions beyond aqueous solutions could also be used to separate NPs.

3.1.1 Gradient Media

Media applied in density gradient centrifugation includes small hydrophilic molecular organic compound (ethanol, ethylene glycol, glycerin, etc.), macromolecule organic compound (polysaccharide, protein, etc.), alkali metal salts (Cs, K, Rb, Na

salts, etc.), colloidal silica (Percoll, Ludox, etc.), and three organic toluene derivatives (covering Metrizamide, Nycodenz, Iodixanol, etc.) [1].

Clearly, solution prepared from aforementioned media differs in density, viscosity, concentration, chemical composition, and properties. As a result, solution made from one to two kinds of media chosen above can be used to separate colloidal NPs. Before separation, several important points should be taken into consideration when choosing the ideal gradient media:

- ① For the nanoparticles to be separated, the gradient media should be inert to colloidal samples under ambient condition. Moreover, it had better has no toxicity.
- ② The gradient media ought to keep the dispersibility of the separation system without causing agglomeration in the suspension.
- ③ The physical and chemical properties of gradient media should be known, such as the exact density, viscosity, and the specific mixed ratio of the gradient media.
- ④ The gradient media should be pH neutral, and its ionic strength should be low enough to avoid possible aggregations of the colloidal NPs.
- ⑤ The gradient media should be colorless which absorb out of the UV range of the spectrum (i.e., <400 nm).
- ⑥ The gradient media should be easily removed from the separated particles without loss of the activity of the sample.
- ⑦ The centrifugal media should possess bactericidal action, remain its separation characteristics after separating, and do not corrode centrifugal tube, the rotor, and other relevant equipment.
- ⑧ The gradient media should be pure substance with low price.

The above are the requirements of the ideal gradient media, while no single liquid can meet all these criteria. In practical work, the selection of proper gradient media should be considered based on the target structure of NPs [2].

3.1.2 Commonly Applied Gradient Media

3.1.2.1 Aqueous Gradient Media

Water is by far the most widely investigated polar solvent and is described as the universal solvent for its capability to dissolve majorities of substance, environmentally benignity, earth-abundant storage, and low cost. Factually, quite a great number of hydrophilic media constitute the aqueous gradient system with the maximum density range of 1.4 g/cm³. In the pursuit of superior separation effect of various inorganic nanoparticles in aqueous circumstances, sucrose, glycerol, and iodinated compounds are extensively utilized.

Table 3.1 Effect of temperature on the viscosity of sucrose solution

| Concentration (mass concentration)/% | Viscosity/mPa·s | | | | |
|--------------------------------------|-----------------|-------|-------|-------|-------|
| | 0 °C | 5 °C | 10 °C | 15 °C | 20 °C |
| 20 | 3.77 | 3.14 | 2.64 | 2.55 | 1.95 |
| 30 | 6.67 | 5.42 | 4.48 | 3.76 | 3.19 |
| 40 | 14.58 | 11.45 | 9.16 | 7.46 | 6.16 |
| 50 | 44.74 | 33.16 | 25.17 | 19.52 | 15.42 |

Sucrose

Sucrose solution is the most universally used aqueous gradient medium by far, along with its cost-effective virtue. Sucrose is easily soluble in water, and the density range can be tuned from 1.00 to 1.32 g/cm³ and could be further enlarged by adding sodium or potassium bromide, citrate, or tartrate into the solution. Generally, it is easy to prepare sucrose solutions in a variety of buffer systems, and the concentration of sucrose solutions has a definite relationship with its viscosity, density, and refractive index. With the assistance of sucrose gradient, tremendous colloidal nanomaterials have been separated by diameter, including magnetic NPs (cobalt ferrite and manganese ferrite), CuInS₂ quantum dots, graphene [3–5].

Furthermore, the viscosity of sucrose solutions is highly dependent on the temperature and concentration (as shown in Table 3.1) [6]. Increasing viscosity of the sucrose solutions can be reduced by replacing H₂O with D₂O which has a density of 1.11 g/cm³.

Commercially available sucrose generally contains several impurities, so sucrose should be purified first to ensure that it is suitable for density gradient work. Moreover, specially refined sucrose with high purity is broadly available.

Glycerol

Glycerol is miscible with water and easy to volatilize, so that it can be directly removed after the separation. Glycerol is half of sucrose in density and is convenient to prepare solutions with high concentration. Usually, 10–30% linear glycerol gradient can be used as an alternative to the 5–20% linear sucrose gradient for the separation.

However, physio-chemical characteristics of glycerol are less well known compared with sucrose, and the high viscosity of glycerol hinders it from wide use for density gradient.

Iodinated Compounds

Iodinated organic compounds, which possess merits such as low toxicity, low osmolality, high solubility, and high density, were firstly employed as density gradient in 1953 by Holter's group. Compared to other gradient media, it is generally believed that the triiodobenzoic derivatives should be the most ideal and widely adapted gradient media up to now.

Non-ionic fraction of the triiodobenzene derivatives containing Metrizamide, Nycodenz, and iodixanol is suitable for the density gradient separation of all particles due to excellent compatibility and stability for NPs. Compared with the above, iodixanol (C₃₅H₄₄I₆N₆O₅) has a relatively larger formula weight and lower

viscosity; thus, its density gradient is readily formed and most widely used in separating carbon-based materials [7–13]. Isosmotic solution with diverse concentrations can be prepared using 60% w/v iodixanol aqueous solution (purchased as Optiprep™, 1.32 g/cm³).

Ethanol

Ethanol (EtOH), which is also called alcohol or ethyl alcohol, is the principal type of alcohol found in alcoholic beverages. As well known, it is a volatile, flammable, colorless liquid with a slight characteristic odor.

Solutions of ethanol in water with different volume ratios (e.g., 20, 30, 40, and 50%) can be used as the density gradient, which, more importantly, can even provide the hydrophilicity difference to capture NPs with different hydrophilicity/polarity. For instance, Deng et al. [14] developed a hydrophilicity gradient ultracentrifuge separation technique based on solubility/stability difference of carbon nanodots (CDs) in gradient of ethanol/water solutions to separate CDs.

Ethylene Glycol

Regarded as the simplest diol, ethylene glycol (EG) is moderately toxic and miscible with water. The density gradient based on different concentrations of EG/water solutions has been widely applied to separate NPs, such as Ag nanoplates (NPTs) [15], Pd nanoplates, and the Au nanoparticles [16]. Besides, Chang et al. [17] successfully sorted the colloidal MgAl LDHs nanosheets and NiAl LDHs nanosheets by their lateral size using the EG aqueous gradient, indicating the potential of extending the density gradient ultracentrifugation separation (DGUS) method for the further study of structure, composition, and properties of multi-component nanomaterials.

Inappropriate Media for Nanoseparation:

Alkali Metal Salts

In addition to the above mentioned, gradients using alkali metal salts, including sodium bromide (NaBr), sodium iodide (NaI), cesium chloride (CsCl), cesium bromide (CsBr), are widely used for the separation of biological materials (e.g., DNA, RNA, nucleic acid), while for the separation of inorganic NPs there are only a few cases using the CsCl solution [18–20] which can reach 1.92 g/cm³ in density as a gradient media, since salts in density gradient media will reduce the electrostatic stability of charged NPs in liquid solutions and induce the aggregation [16].

3.1.2.2 Organic Gradient Media

The separation of biomolecules usually needs strict osmotic and viscous liquid environment, namely aqueous gradient. While a lot of nanoparticles were synthesized in the organic phase and got solubilized therein (such as Au, CdSe, and Si nanocrystals), phase transfer might cause serious aggregation. In addition to extending the range of colloidal systems which can be separated, there are several other advantages of using an organic density gradient rather than the conventional aqueous gradients. To begin with, combining synthesis optimization and separation together can prepare samples that cannot be prepared by synthesis optimization

alone. Secondly, colloidal NPs synthesized and dispersed in an organic medium can be directly separated after synthesis without transferring to an aqueous medium, which avoids the possible aggregation and clustering of NPs under non-optimized conditions. Thirdly, since the density gradients are composed of organic solvents without any solid additives, the solvents can be evaporated without leaving any residue, to get “pure” sample. Colloidal NPs with constant size distribution can thus be captured in the gradient together with polymers.

Cyclohexane/Carbon Tetrachloride

Cyclohexane is a cycloalkane with the molecular formula of C_6H_{12} and a normal density of 0.779 g/cm^3 , which is miscible with various organic solvents and frequently used as a non-polar organic gradient media in density centrifugation. Carbon tetrachloride (CCl_4) is a colorless and volatile liquid with a slight sweet smell and a normal density of 1.595 g/cm^3 . The density gradient based on the combination of non-polar cyclohexane and CCl_4 can realize a large density range, which is ideal for the separation of NPs [21–23].

Chlorobenzene/2,4,6-Tribromotoluene

Chlorobenzene is a colorless liquid with a density of 1.484 g/cm^2 , and the mixture of chlorobenzene and 2,4,6-tribromotoluene (TBT) is usually used as a density gradient to separate carbon nanotubes [24] and monodisperse silicon nanocrystals [25].

Other macromolecular organics such as Ficoll and dextran are also suitable for fractionating biological materials, while the difficult handling procedure hinders the applications due to their relative low density and high viscosity.

3.2 Choice of Density Gradient

In order to obtain the maximum resolution, the suspension samples on gradient should be as thin as possible. In a given centrifugal field, when particles move through the density gradient, the buoyancy density and viscous resistance should be minimal, and the gradient density range should be small [26]. In this case, the centrifugal separation time will be shorter and there will be minimum diffusion effect. Gradients can be divided into continuous and discontinuous (step) gradients, as illustrated in Fig. 3.1.

3.2.1 Step Gradient

Discontinuous (step) density gradient is primarily restricted to isopycnic separations where it obtains sharper bands.

The preparation method for step gradients is quite simple. A series of solutions with increasing density are prepared, and orderly add these solutions to the centrifuge tube or zonal rotor by using the liquid transfer tube, syringe, or density

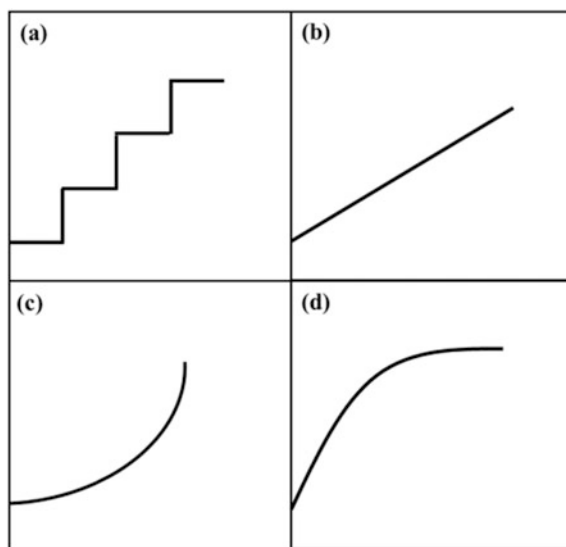


Fig. 3.1 Models of different types of gradients. Step gradients **a** are used only for isopycnic separations. Continuous gradients are **b** linear, **c** convex, **d** concave and could be used for rate-zonal separations or isopycnic separations

gradient pump. According to the operation sequence, it can be divided into overlayering and underlayering method.

Overlayering Method

Overlayering method is the method to prepare gradients simply by adding solutions from bottom to up, starting with the highest concentrated solution first. After adding the bottom layer, the subsequent solutions should be added as per the following instructions: (1) The centrifugal tube should be kept upright, and the solutions should be syringed enough slowly along the tube wall to the lower part. (2) The long needle tip can be bent into “L” shape, and the upper surface of the lower layer is covered by solution with low density. (3) A gradient instrument, such as DCF-U-type gradient instrument and can be used to track the liquid concentration.

Typically, for the preparation of a three-layer discontinuous gradient with the density of each layer of 1.2, 1.4, 1.6, and 1.8 g/cm^3 , the solution with greatest density (1.8 g/cm^3) should be added to the bottom of the centrifuge tube according to the required volume, and then the solutions should be added with 1.6, 1.4, 1.2, and 1.2 g/cm^3 on the top step by step (Fig. 3.2).

Underlayering Method

The underlayering method is the method to prepare gradients by underlayering the required solutions one under the other, starting with the lightest solution first. A long and thin tube or needle can be inserted into the bottom of a centrifuge tube,

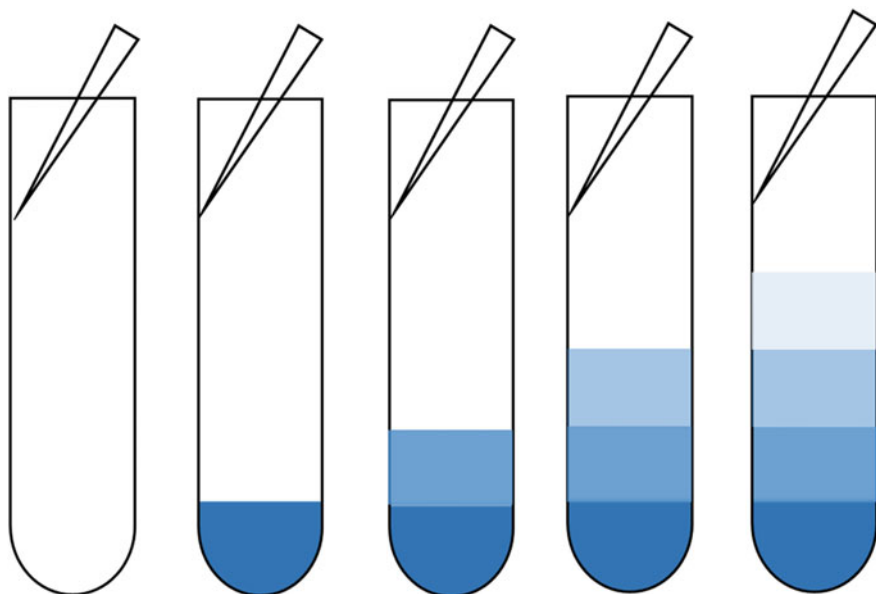


Fig. 3.2 Scheme of preparing density gradient via upper-spread method

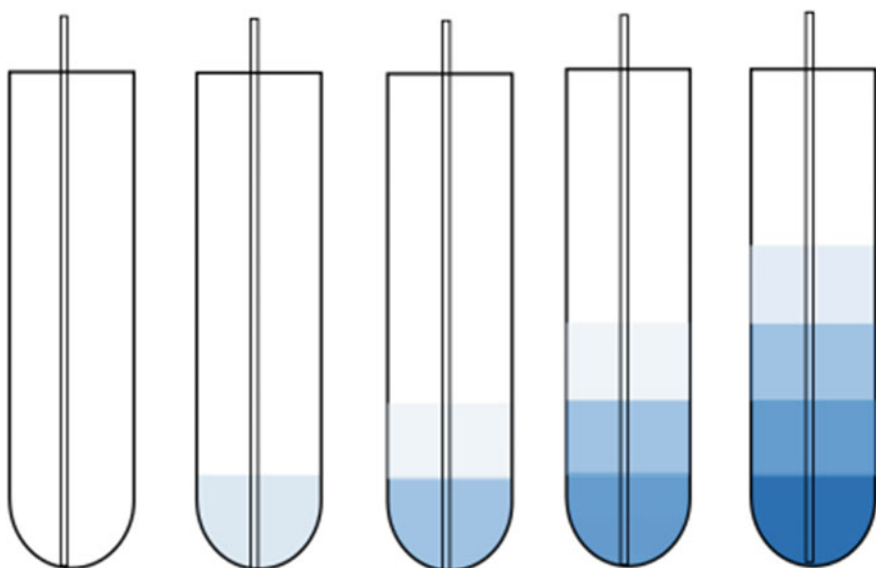


Fig. 3.3 Scheme of preparing density gradient via bottom-spread method

as shown in Fig. 3.3. The preparation process should be very careful, and the needle cannot be shaken and has to be maintained vertically, so as not to have a large impact on each layer.

It is worth noting that bubbles should be avoided during the gradient preparation. Too many bubbles will inevitably interfere the interfaces between the layers. Further, after layering the step gradient, it is necessary to check that there are sharp, refractive interfaces between each of the solutions to ensure that the gradient has been made correctly.

Once prepared, the step gradient should be used immediately; otherwise, it would probably transform into continuous gradient over several hours owing to solvent diffusion. If required, the sharp steps can be “rounded” by leaving the gradient standing vertically for about 2 h.

3.2.2 *Continuous Gradient*

The density of continuous gradient varies smoothly and continuously, which are usually linear or exponential, and has been advocated for particular separations. Besides, gradients with more complex shapes are only used for centrifugal separations in zonal rotors.

The shapes of the continuous density gradient can be designed as linear, concave, or convex curve type (Fig. 3.1) according to the characteristics and requirements of samples. Among them, the linear gradient is the most common instance which could be prepared in the following strategies.

Diffusion Method

A linear gradient placed for a period of time can be formed with diffusion. This method starts with preparing a step gradient whose density interval should be about 1–2 cm thickness of each layer of liquid and the relative linear gradient ought to be formed through free diffusion in a vibration-free condition.

Since the time required for diffusion varies according to the diffusion coefficient of gradient materials, it would be better to select the gradient media of low molecular weight. In addition, the difference of temperature, concentration, viscosity, thickness, and density of each layer has certain influence on the time to form a gradient. In order to shorten the diffusion time, a wire ring can be used by moving along the wall up and down for several times [6]. The diffusion method can stabilize the sample zone, which is suitable for the coarse separation process. However, it is not strict for quantitative analysis and time-consuming.

Gradiometer Method

A linear gradient can be easily prepared using an apparatus, as shown in Fig. 3.4. It is composed of two chambers with an outlet and a stirrer in the mixing chamber while the other chamber is the reservoir. The gradient is prepared with two different

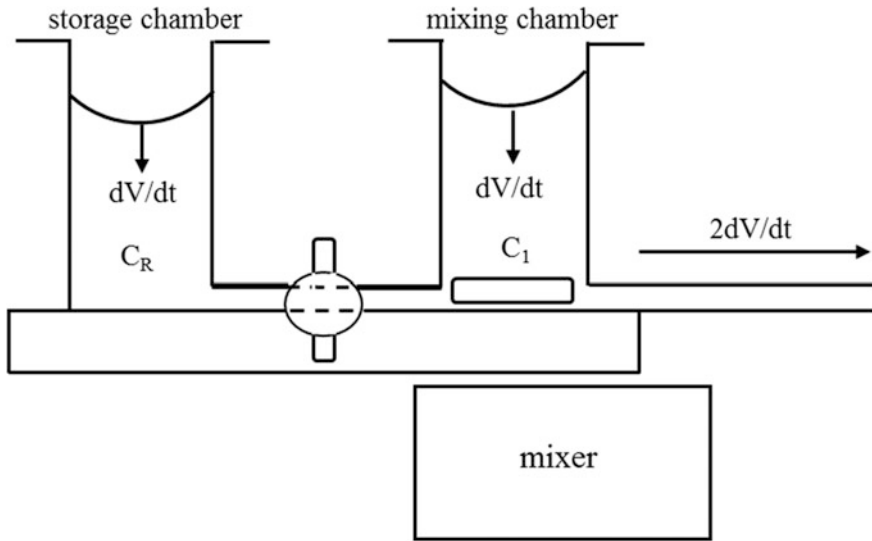


Fig. 3.4 Scheme of a simple two-cylinder gradient apparatus [6]

concentrations of solution, a high-density solution (the heavy solution) and a solution of low density (the light solution). The equal volume of light solution and heavy solution was added into two chambers, and a solution flows at a rate of dV/dt (V is the volume, t is the time) from one room to the other one. The mixed liquid flows out at a rate $2dV/dt$.

The concentration of the solution from the mixing chamber can be calculated by using Eq. 3.1.

$$C_t = C_M + (C_R - C_M)V_t/V_1 \quad (3.1)$$

where C_t is the concentration of the liquid outflows from the mixing chamber at time " t ", C_R is the concentration of the liquid in the storage chamber, C_M is the initial concentration of the liquid in the mixing room, V_t is the volume of the liquid flows out at the time " t ", and V_1 is the original volume of the liquid in each room.

If C_R equals the concentration of the gradient light edge, V_0 becomes the final volume of the gradient, so that the bigger C_M is, the larger step the gradient has.

The formation of linear gradient is assumed simple, but, in fact, it is rather difficult to obtain real linear gradient. In order to gain a satisfactory linear gradient, the following conditions must be achieved: The two rooms must have the same geometric shape, and their respective liquid volumes should be the same at any time as well as reduce at equal rate, so the corresponding device must be utilized to control the flow rate.

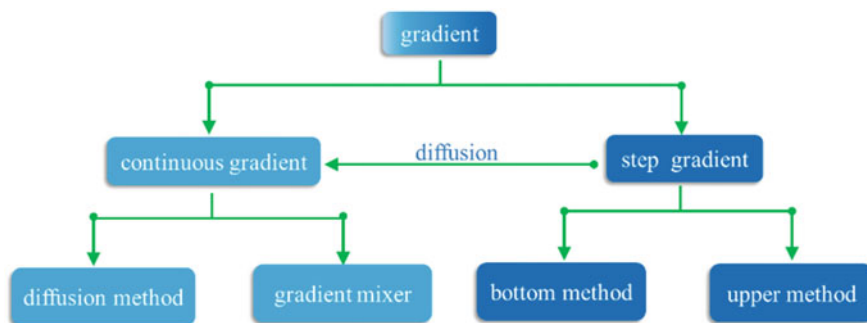


Fig. 3.5 Schematic of the gradient types and relevant preparation methods

As stated previously, the density gradient can be prepared by several methods relying on the type of gradient required and individual preferences. Figure 3.5 schematically presents the category of gradients and preparation methods.

Based on the above analysis, the advantages of discontinuous (step) gradient can be summed up as: (1) simply made without special equipment; (2) high efficiency, 10 min is enough to finish separations in most cases; (3) high repeatability, the gradient solutions remain stable for at least 1 h; (4) adjustable, the density range could be further narrowed down according to practical pre-separation results. Therefore, the step gradient is widely applied for NPs separations.

3.3 Choice of a Rotor Type

Centrifugal rotor is the core component of the centrifuge, and all systems in centrifuge are configured to ensure the rotor under proper conditions. Rotor not only affects the separation effect, but also it is the main stress component of the centrifuge, so the safe operation of the centrifuge is very important. This section mainly introduces the classification of the centrifuge rotor and rotor-type selection.

3.3.1 Classification of Centrifuge Rotors

According to the properties, the centrifugal rotor can be divided into two types: the analysis of the rotor and the preparation of the rotor. Here, we mainly discuss the classification of the rotors. Material, shape, volume, and utility of each rotor are not identical, what we commonly use are fixed-angle rotor, swing-out rotor, zonal rotor, and vertical rotor.

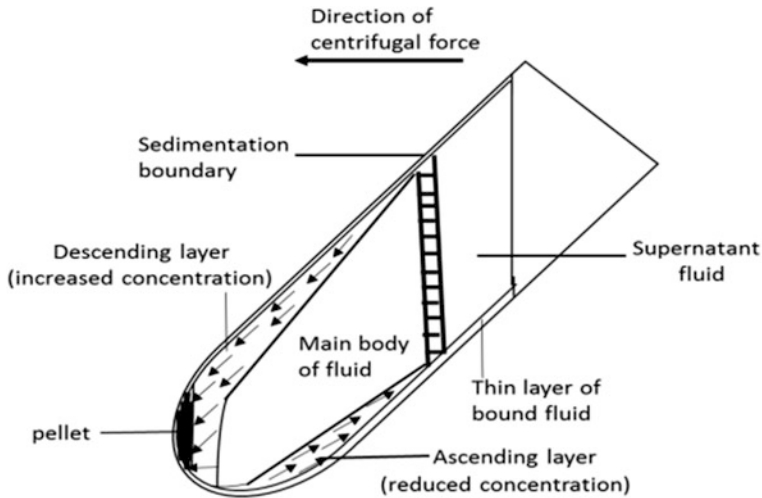


Fig. 3.6 Schematic diagram of a fixed-angle rotor

3.3.1.1 Fixed-Angle Rotors

Fixed-angle rotors (Fig. 3.6), as the name suggests, usually have several centrifugal tube cavities with a fixed angle. Angles between the center of the cavity axis and the axis of rotation range from 14 to 40°. The greater the angle, the better separation effect is.

Under the centrifugal force, particles will settle across the tube until hitting to centrifuge tube wall and then slide down to the bottom of the tube, which is called “wall effect” phenomenon.

The advantage here is that fixed-angle rotor has a large capacity, the center of gravity is low, the operation is balanced, and so fixed-angle rotor has a long lifetime.

The disadvantage is that the wall effect is easy to cause the settling particles to be disturbed by the sudden change of speed.

3.3.1.2 Swing-Out Rotors

Swing-out rotor (Fig. 3.7) usually has four or six free buckets (centrifugal casing); when the rotor is in static status, buckets are hung vertically. When the rotating speed reaches 200–800 rpm per minute, buckets will swing horizontally; this rotor is the best choice for density gradient zone centrifugation.

The advantage is that gradient material can be placed in the vertical centrifugal tube. Unlike the sediment samples in fixed-angle rotor, it is easy to take out the separated sample from the tube after the separation. While the disadvantage is that

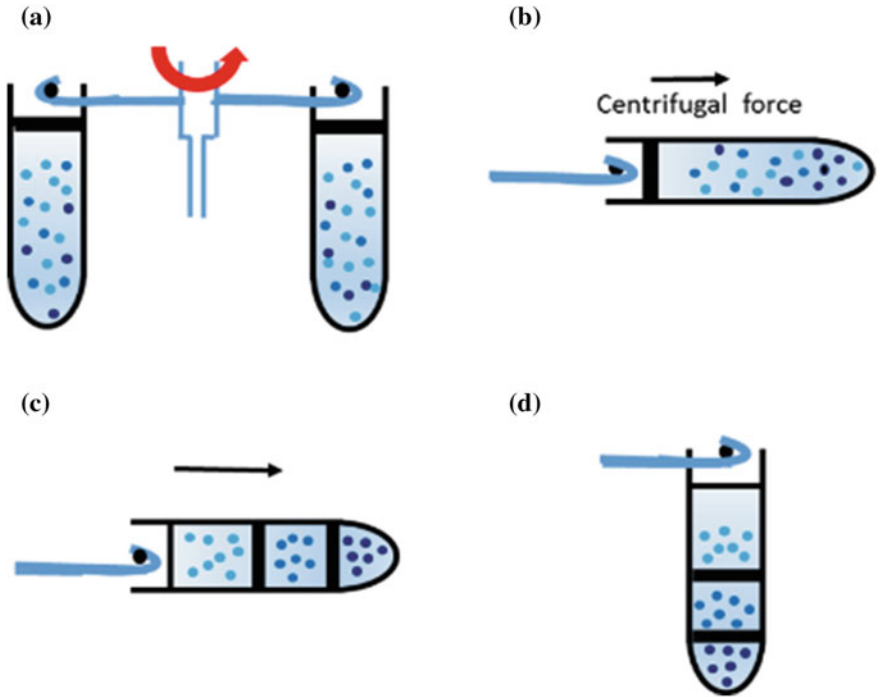


Fig. 3.7 Separations in swing-out rotors. The tubes are filled. Loaded into each bucket and attached to central body of the rotor, at rest the buckets of the rotor hang vertically. **a** As the rotor begins to move, the buckets move out so that they are perpendicular to the axis of rotation. **b** During centrifugation, the particles sediment down the tube. **c** When the rotor come to a stop. **d** The bucket return to a vertical position, and there is no reorientation of the liquid in the tubes

the sedimentation distance of particles is long, correspondingly the centrifugal time will be long.

3.3.1.3 Zonal Rotors

Zonal rotor is mainly composed of a rotor barrel and an unscrewed top cover. Wherein, the rotor barrel is equipped with a cross-shaped clapboard device, which divides the rotor barrel into four or more chambers. The gradient liquid or sample liquid is pumped from the inlet pipe in the center of the rotor and is distributed around the rotor through a conduit inside the clapboard. The clapboard in the rotor can maintain the stability of the sample band and the gradient medium.

The sedimentation situation in zonal rotor differs from fixed-angle rotor or swing-out rotor. Under radial scattering effect of centrifugal force, particle settling distance will be unchanged, so the zonal rotor has a little “wall effect.”

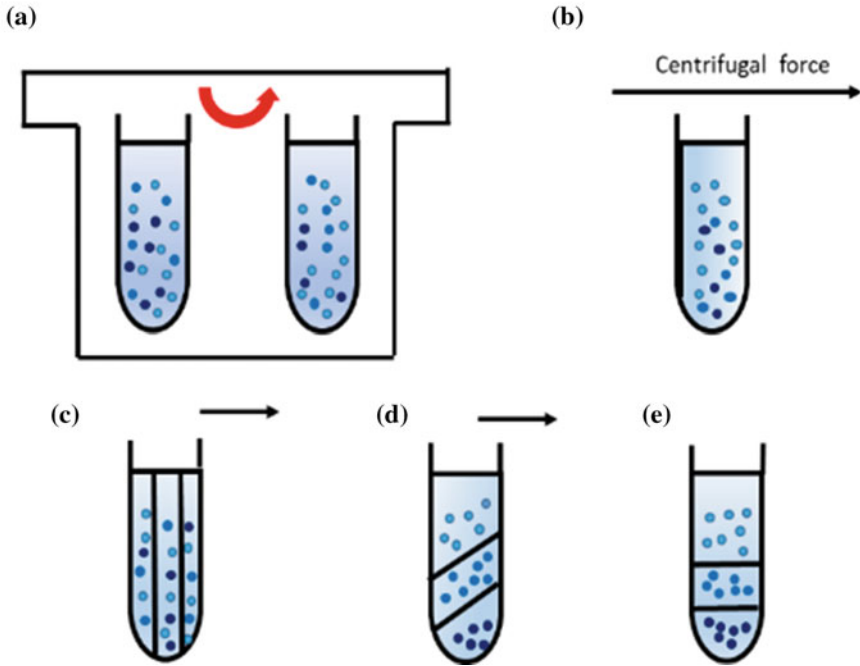


Fig. 3.8 Separation in vertical rotors. The tube is filled completely, sealed, and loaded into the pockets of the rotor (a). As the rotor begins to move, the liquid in the rotor reorientates through 90° (b). And the particles start to sediment across the tube (c). As the rotor decelerates below 1000 rpm, the bands reorientate (d). And as the rotor comes to a stop, this process is completed (e)

The advantage is that zonal rotor can avoid zone and settling particles becoming disorderly, which can also avoid disturbance of zone and settling particles. Furthermore, good separation effect, high speed, easy for gradient recovery, and having no influence on the resolution are the merits. In particular, this method can separate a large quantity of samples at once, about 60 ml. Compared to the chromatographic separation method, this method has great advantage in separation of sample with large capacity.

The disadvantage of zonal rotor is that the direct contact is inevitable between the sample and the medium, the corrosion resistance is high, and the operation procedure is complex.

3.3.1.4 Vertical Rotors

The centrifuge tubes of vertical rotor (Fig. 3.8) in the centrifugal process are kept to be vertical, that is the parallel orientation of the rotating shaft and the centrifuge tube. Compared to the fixed-angle rotor and swing-out rotor, the vertical rotor

shortens the centrifugal time and at the same time improves the separation resolution.

Advantages are listed as following. The vertical rotor is mainly used for isopycnic gradient centrifugation. And because there is no wall effect (at least in the early stage of the gradient), it is also ideal for rate-zonal centrifugation. The difference between the minimum centrifugal acceleration and the maximum centrifugal acceleration of the vertical rotor is not very obvious, and all kinds of particles are in the strong centrifugal field, so the separation speed is fast and the resolution is high.

Disadvantages of vertical rotor are also illustrated here. Due to the redirection of the vertical rotor, the sample band in the centrifuge is wider in the latter part of the centrifuge than in the other rotor samples. Another problem is that the material that precipitates or floats on the gradient will be distributed over the length of the tube, and it can contaminate the supernatant when the centrifuge is complete.

3.3.2 The Selection Principle of the Rotors

Fixed-angle rotor has a relatively short distance for the centrifugal settling and recovery. That is to say, gradient curve flattens and the extension of the distance are conducive to the recycle of particles after centrifugation, so the settling distance of vertical rotor extends greater than the fixed-angle rotor; so vertical rotor is more conducive to isopycnic separation. On the contrary, granular zone width in the swing-out rotor is the narrowest, and the width of vertical rotor is the widest. It must be pointed out to in order to use the fixed-angle rotor for density gradient centrifugation, the separation of sample should not be too much, centrifugal speed is unfavorable and exorbitant, separation time should not be too long, otherwise, the precipitation will be attached to the wall, and the following causes pollution and influences the separation effect.

Swing-out rotor has the longest particle settling path, from meniscus to the bottom of the centrifuge tube. It also has merits including a wide range of density change toward to distance, easy to keep a wide gap between particles zones, can effectively prevent the formation of mixture, suitable for the separation and purification of larger virus particles and subcellular particles, but the swing-out rotor forming gradient costs a long time and, as a result, low efficiency of centrifugal.

Zonal rotor has the largest volume, which can separate about 60 mL samples once, so it is convenient for large quantity separation. Moreover, this method can avoid disorder of zone and settling particles and ensure good separation efficiency.

Vertical rotor has the shortest settling distance, centrifugal time, and the highest centrifugal efficiency, which is suitable for the extraction and purification of small particles. Due to the reorientation of liquid gradient medium in the centrifugal process and the centrifugal stop, the sample which is easy to produce “attached wall” is not suitable. And due to the circulation mixing and certain requirements for

the concentration of the gradient, the viscosity of the medium, the use of fixed-angle rotor has a certain limit in the density gradient centrifugation.

3.3.3 The Relationship Between the Type of the Rotor and Centrifugal Force

In the ultracentrifuge, the specific rotor type corresponds to a specific speed range, and the specific speed relates to centrifugal force. Therefore, in different systems, the choice of rotor type is actually the choice of centrifugal force. It is well known that the sedimentation speed of the particles goes up as the centrifugal force increases. Thus, more rapid separation can be achieved by increasing centrifugation speed. Here, we briefly introduce some of the concepts of centrifugal force and the relationship between sedimentation speed and centrifugal force.

3.3.3.1 Centrifugal Force

At a certain angular velocity, any object that has a circular motion is subjected to an outward centrifugal force. The size of centrifugal force (F_c) is equal to the product of the centrifugal acceleration ($\omega^2 X$) and the particle mass (m), that is:

$$F_c = m\omega^2 X \quad (3.2)$$

The ω is the rotating angular, unit in radians/second; X is the distance from the center of the rotation to the particles; use cm as a unit; m is the mass in (g).

It can be seen that the size of the centrifugal force is proportional to the square of the rotational speed and the radius of rotation. Under certain conditions, the more far away from the axis of the particle, the greater the centrifugal force is. In the process of centrifugation, as the particles move in the centrifuge tube, the centrifugal force is also changed.

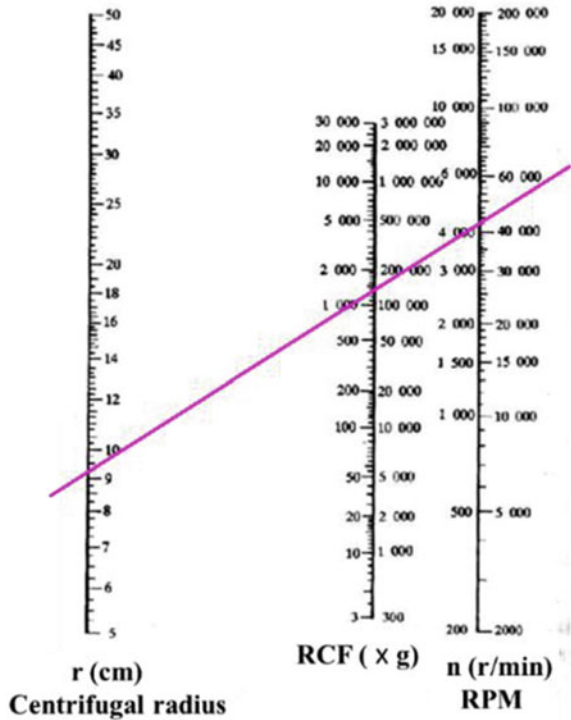
In practical work, the applied centrifugal force data is the mean data of centrifugal force which distributed throughout the centrifugal tube. That is, particles are affected by centrifugal force at the midpoint of the centrifugal solution.

3.3.3.2 Relative Centrifugal Force (RCF)

Commonly used “relative centrifugal force” or “digital * G” indicates that the centrifugal force, as long as the RCF value is constant, a sample can get the same centrifugal results under different centrifuge.

RCF is the actual centrifugal field that is converted to multiples of acceleration of gravity. That is:

Fig. 3.9 Conversion of speed and relative centrifugal force



$$RCF = \frac{F_{Centrifugal\ force}}{F_{Gravity}} = \frac{m\omega^2 X}{mg} = \frac{\omega^2 X}{g} = \frac{2\pi n X}{60/980} = 1.118 \times 10^5 \times X \times n \quad (3.3)$$

The X is the distance from the center of the rotation to the particles; use cm as a unit and n on behalf of the centrifuge speed (rpm/min).

3.3.3.3 The Selection of Speed (N) and Relative Centrifugal Force (RCF)

As shown in Fig. 3.9, the “ r ” is the distance from the middle of the centrifuge tube to the center of the centrifuge shaft center (cm) and “ n ” on behalf of the centrifuge speed (rpm/min).

If the centrifuge speed needs to be converted to centrifugal force, firstly a known radius should be picked up in the “ r ” scale and the centrifugal speed should be taken in the “ n ” scale and extends to the RCF scale; intersection of lines is the corresponding value of the centrifugal force. If RCF is known, centrifuge speed can also be got.

In order to make specific rotor centrifugal conditions and get the same separation effect on another type of rotor, the relationship between the relative centrifugal force and the centrifugal time should be considered.

$$T_1 RCF_1 = T_2 RCF_2 \quad (3.4)$$

3.4 Choice of an Appropriate Separation Speed and Time

As discussed in the introduction of Sect. 3.2.1, we know that the density gradient centrifugation mainly includes three ways: differential centrifugation, isopycnic centrifugation, and rate-zonal centrifugation. With different choices of various methods, the calculation of the centrifugal time is also different.

In general, due to the changing shape of the gradient, the separation time of rate-zonal centrifugation is no more than 2 h. In terms of isopycnic centrifugation, it takes at least 9 h for the samples to be separated thoroughly. There is no clear statement for the separation time between 2 and 9 h, and the exact time depends on the specific samples. The general rule is that with the speed increases, the less time-consuming.

3.4.1 Differential Centrifugation

For differential centrifugation, the centrifugal time is the time when a particle is completely settled to the bottom of the tube. It is different from the isopycnic centrifugation or rate-zonal centrifugation; the influencing factors on centrifugal time of differential centrifugation are not complex, and the settling time can be obtained through calculation.

K coefficient is a characteristic parameter to characterize the efficiency of rotor centrifugal separation. The K coefficient of the rotor is taken into account in the centrifugal distance and the maximum centrifugal acceleration, when $S_{20,w}$ is known (sedimentation coefficient of water at 20 °C); the K coefficient can be used to calculate the centrifugal time of the maximum speed.

$$t = \frac{K}{S_{20,w}} \quad (3.5)$$

Therefore, if the K coefficient of a rotor and $S_{20,w}$ value are known, then the centrifugal time can be calculated at the maximum speed.

Among them, the K coefficient is related to the centrifugal distance and rotational speed of the rotor.

$$K = \frac{(\ln r_2 - \ln r_1)}{\omega^2} \times \frac{10^{13}}{3600} \quad (3.6)$$

In the above formula, speed of revolution $\omega = 0.10472n(\text{r/min})$; r_1 : the distance from the center of the rotating shaft to the liquid level of the sample (cm); r_2 : the distance from the center of the rotating shaft to the bottom of the centrifuge tube (cm).

Practical Application

Chen and co-workers reported the isolation of Au nanoparticle dimers in high purity by exploiting the high density of CsCl solutions. There is a typical setup of differential centrifugation, where 62% of CsCl and 11% of Au nanoparticle clusters with polystyrene-block-poly (acrylic acid) (AuNPn@PSPAA) in water were layered from bottom to top. To partition the AuNPn@PSPAA, a concentrated nanoparticle solution was carefully overlaid atop the 11 + 62% CsCl gradient, and the solution was then centrifuged in a desktop microcentrifuge at 8500 rpm (5800 g) for 20 min. The resulting solution showed two distinct bands of red and purple color, separated by a gap. The sample was extracted, purified to remove the excess CsCl, and then got the Au nanoparticle dimers with high purity [28].

3.4.2 Isopycnic Centrifugation

For isopycnic centrifugation, the time of centrifugation is equivalent to the time when the particles reach the point of equal density completely.

In most of the equilibrium gradient centrifugation, what added to the centrifuge tube is the mixture that evenly mixed with the sample and gradient medium. As long as the centrifugal time is long enough, the centrifugal gradient medium would form equilibrium gradient under the action of centrifugal force; at the same time, samples exist in each settling or floating, which close to their density, and finally the sample zone is formed. In the centrifuge, large particles reach the equilibrium position very quickly, and the smaller particles take a longer time. Therefore, the estimated time required to reach the equilibrium position of the particle is the standard time for small particles. Through the next calculation:

$$T = \frac{9.8310^{13} \beta^0 (\rho_p - 1)}{N^4 r^2 S_{20, w}} \quad (3.7)$$

In the above formula, N is the actual speed, r/min; ρ_p stands for the buoyant density of samples; r is the distance from the center of rotation to the sample zone center, cm; β^0 see β^0 coefficient table; $S_{20, w}$ is the sedimentation coefficient for the sample at 20 °C, water as the medium.

Practical Application

Christine M. Nolan et al. separated the PEG cross-linked poly(N-isopropylacrylamide) microgel particles using isopycnic centrifugation in sucrose medium. The sucrose density gradient equilibrium centrifugation studies were carried out using a Beckman ultracentrifuge with 10-mL ultraclear centrifuge tubes. A sucrose density gradient was made by carefully layering 25, 20, 15, and 10% sucrose solutions (2.0 mL each) within the centrifuge tubes and then depositing 0.03 mL of the PEG cross-linked pNIPAm microgel dispersion on top. The samples were then centrifuged at 26 °C and 25,000 rpm for 4 h to allow the particle bands to reach their equilibrium density zones. Actual densities of these particles were not calculated, but qualitative differences were obtained by comparing the distances travelled down the sucrose density gradient. Samples were prepared in triplicate and presented as an average value ± 1 standard deviation [27].

3.4.3 Rate-Zonal Centrifugation

For rate-zonal centrifugation, centrifugal time is the time of the formation of a well-defined zone.

Unlike differential centrifugation, there are many factors that affect the separation effect of particles in rate-zonal centrifugation. Due to the big difference between $\rho_{\text{Tsolv}}, \eta_{\text{Tsolv}}$ and $\rho_{20,w}, \eta_{20,w}$, even in the top of the gradient, and the $\rho_{\text{Tsolv}}, \eta_{\text{Tsolv}}$ is continuous changing, the separation requirements for particle centrifugation speed and time are affected not only by the influence of particle size, but also by gradient density range, the gradient shape, and concentration. Therefore, it is very difficult to determine the centrifugal state of the rate-zonal centrifugation. The most commonly used are the following two methods of estimation.

3.4.3.1 $\omega^2 t$ Calculation Method

This approach is to divide gradient into a series of small radius of components along the direction of the centrifugal force field. With the average density (ρ_M) of each increment of radius and viscosity (η_M) to calculate the time that the particle through the incremental required, the total of centrifugal effect is computed as:

$$\omega^2 t = \frac{(\rho_p - \rho_M)}{S_{20,w} \eta_{20,w}} \sum_{r_1}^{r_2} \frac{\eta_M \Delta r}{(\rho_p - \rho_M) r} \quad (3.8)$$

In the above formula, ρ_M and η_M refer to the density and viscosity of the center of each gradient in radius; r is the radius of increment center; r_1 and r_2 refer to the radius of the distance of the particle zone at the beginning and end of the operation, respectively.

Table 3.2 K' coefficient of some rotors

| Rotor | The highest speed(r/min) | K' coefficient | | | |
|-----------------------|--------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | | 1.2 g/cm ³ | 1.4 g/cm ³ | 1.6 g/cm ³ | 1.8 g/cm ³ |
| <i>Beckman</i> | | | | | |
| SW60Ti | 60,000 | 160 | 135 | 128 | 124 |
| SW41Ti | 41,000 | 379 | 317 | 300 | 292 |
| SW25.1 | 25,000 | 1035 | 867 | 822 | 799 |
| <i>Dupont/Sorvall</i> | | | | | |
| AH627 | 27,000 | 816 | 683 | 646 | 629 |
| TST60.4 | 60,000 | 301 | 252 | 239 | 232 |
| TH41 | 41,000 | 420 | 353 | 334 | 325 |
| TV-850 | 50,000 | 115 | 95 | | |

The calculation process of the method is complex, so it is rarely used in actual work, but the $\omega^2 t$ calculation method with many applications is applied in centrifugal experiment.

3.4.3.2 K' Coefficient Estimation Method

In the rate-zonal centrifugation, the K' coefficient is used to estimate the time of particle precipitation, and the equation is as follows:

$$t = \frac{K'}{S_{20,w}} \quad (3.9)$$

In the formula, t is the centrifugal time; $S_{20,w}$ refers to particle sedimentation coefficient in standard condition; K' for the rotor's clarification coefficient; its value depends on the gradient, temperature, and particle density.

Table 3.2 lists some of the K' coefficients of the rotor, which is a linear sucrose gradient of 5–20%, with a temperature of 5 °C. With the K coefficient of each circumstance is close to each other, the lower K' coefficient is, the higher separation efficiency is.

Practical Application

George M. Whitesides et al. created a new approach to size-dependent and shape-dependent separation of nanoparticles through rate zonal centrifugation using multiphase systems as separation media. They use this method to separate the reaction product (nanorods) and byproducts (nanospheres and bigger particles) of a synthesis of gold nanorods. After estimation, they found the RCF must be greater than 8,800 g for a 25-nm (diameter) gold nanosphere ($d_{\text{gold}} = 19.3 \text{ g/cm}^3$) in order to overcome the effect of the interfacial surface energy. They chose to use 16,000 g to ensure that they were well beyond the limit and also to reduce the time required to separate the objects [28].

References

1. Sartory WK (1970) Fractional cleanout in a continuous-flow centrifuge. *Sep Sci Technol* 5 (2):137–143
2. Perardi TE, Leffler RAA, Anderson NG (1969) K-series centrifuges II. Performance of the K-II rotor. *Anal Biochem* 32(3):495–511
3. Prantner AM, Chen J, Murray CB et al (2012) Coating evaluation and purification of monodisperse, water-soluble, magnetic nanoparticles using sucrose density gradient ultracentrifugation. *Chem Mater* 24(21):4008–4010
4. Sun X, Luo D, Liu J et al (2010) Monodisperse chemically modified graphene obtained by density gradient ultracentrifugal rate separation. *ACS Nano* 4(6):3381–3389
5. Speranskaya ES, Beloglazova NV, Abé S et al (2014) Hydrophilic, bright CuInS₂ quantum dots as Cd-free fluorescent labels in quantitative immunoassay. *Langmuir* 30(25):7567–7575
6. Pine GG, Yuan XL (2008) Modern separation science and technology series: centrifugal separation (M). Chemical Industry Press, Beijing
7. Kirkland J, Yau W, Doerner W, Grant J et al (1980) Sedimentation field flow fractionation of macromolecules and colloids. *Anal Chem* 52(12):1944–1954
8. Sun X, Zaric S, Darancioglu D et al (2008) Optical properties of ultrashort semiconducting single-walled carbon nanotube capsules down to sub-10 nm. *J Am Chem Soc* 130(20):6551–6555
9. Green AA, Hersam MC (2007) Ultracentrifugation of single-walled nanotubes. *Mater Today* 10(12):59–60
10. Green AA, Duch MC, Hersam MC (2009) Isolation of single-walled carbon nanotube enantiomers by density differentiation. *Nano Res* 2(1):69–77
11. Green AA, Hersam MC (2009) Solution phase production of graphene with controlled thickness via density differentiation. *Nano Lett* 9(12):4031–4036
12. Ghosh S, Bachilo SM, Weisman RB (2010) Advanced sorting of single-walled carbon nanotubes by nonlinear density-gradient ultracentrifugation. *Nat Nanotechnol* 5(6):443–450
13. Liu J, Hersam MC (2010) Recent developments in carbon nanotube sorting and selective growth. *MRS Bull* 35(4):315–321
14. Deng L, Wang X, Kuang Y et al (2015) Separation of non-sentimental carbon dots using “hydrophilicity gradient ultracentrifugation” for photoluminescence investigation. *Nano Res* 8 (9):2810–2821
15. Zhang C, Luo L, Luo J et al (2012) A process-analysis microsystem based on density gradient centrifugation and its application in the study of the galvanic replacement mechanism of Ag nanoplates with HAuCl₄. *Chem Commun* 48(58):7241–7243
16. Bonaccorso F, Zerbetto M, Ferrari AC et al (2013) Sorting nanoparticles by centrifugal fields in clean media. *J Phys Chem C* 117(25):13217–13229
17. Chang Z, Wu C, Song S et al (2013) Synthesis mechanism study of layered double hydroxides based on nanoseparation. *Inorg Chem* 52(15):8694–8698
18. Chen G, Wang Y, Tan LH et al (2009) High-purity separation of gold nanoparticle dimers and trimers. *J Am Chem Soc* 131(12):4218–4219
19. Wang Y, Chen G, Yang M et al (2010) A systems approach towards the stoichiometry-controlled hetero-assembly of nanoparticles. *Nat Commun* 1:87
20. Yang M, Chen T, Lau WS et al (2009) Development of polymer-encapsulated metal nanoparticles as surface-enhanced Raman scattering probes. *Small* 5(2):198–202
21. Bai L, Ma X, Liu J et al (2010) Rapid separation and purification of nanoparticles in organic density gradients. *J Am Chem Soc* 132(7):2333–2337
22. Ma X, Kuang Y, Bai L et al (2011) Experimental and mathematical modeling studies of the separation of zinc blende and wurtzite phases of CdS nanorods by density gradient ultracentrifugation. *ACS Nano* 5(4):3242–3249
23. Song S, Kuang Y, Liu J et al (2013) Separation and phase transition investigation of Yb³⁺/Er³⁺ + co-doped NaYF₄ nanoparticles. *Dalton T* 42(37):13315–13318

24. Stürzl N, Hennrich F, Lebedkin S et al (2009) Near monochiral single-walled carbon nanotube dispersions in organic solvents. *J Phys Chem C* 113(33):14628–14632
25. Mastronardi ML, Hennrich F, Henderson EJ et al (2011) Preparation of monodisperse silicon nanocrystals using density gradient ultracentrifugation. *J Am Chem Soc* 133(31):11928–11931
26. Guglielmi L, Battu S, Le Bert M, Faucher JL, Cardot PJP, Denizot Y (2004) Mouse embryonic stem cell sorting for the generation of transgenic mice by sedimentation field-flow fractionation. *Anal Chem* 76(6):1580–1585
27. Nolan CM, Reyes CD, Debord JD, García AJ, Lyon LA (2005) Phase transition behavior, protein adsorption, and cell adhesion resistance of poly(ethylene glycol) cross-linked microgel particles. *Biomacromol* 6(4):2032
28. Akbulut O, Mace CR, Martinez RV, Kumar AA, Nie Z, Patton MR, Whitesides GM (2012) Separation of nanoparticles in aqueous multiphase systems through centrifugation. *Nano Lett* 12(8):4060–4064