CHITOSAN-BASED HYDROGELS

Functions and Applications

Edited by

Kangde Yao • Junjie Li
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Preface

Chitosan is a deacetylated derivative of chitin, which is an abundant marine resource that can be obtained from the shells of shrimps or crabs. Chemically, it is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). It is a unique natural base polysaccharide whose structural units are similar to those of glycosaminoglycans of the extracellular matrix (ECM). As a result, chitosan has the advantage of being multiply bioactive, which can be taken advantage of in constructing biomaterials of different applications. At the same time, a large number of active hydroxyl and amino groups are available within chitosan molecules. In order to produce various chitosan derivatives, these groups can be set as target sites for chemical modification. Chitosan molecules undergo different types of degradation reactions that result in different degradation products with different molecular weights, some of which will take part in the synthesis of ECM. To optimize the physical, mechanical, and biological properties of these materials, different kinds of chitosan networks are designed and produced, resulting in chitosan-based biomaterials. These biomaterials are used to make a variety of medicines, drug controlled-release carrier, tissue-engineering scaffolds, and immobilized enzymes. With the development of stem-cell technology, chitosan-based biomaterials are also used as scaffolds for stem cells, which will regulate the proliferation and differentiation of cells. Today, more and more papers and patents on chitosan-based materials are emerging. However, very few of these materials can be merchandised, which means that more work needs to be done to fill the gap between research and production. Recognizing this situation, the authors try to summarize progress in the research and development of chitosan-based biomaterials, which they think will not only inspire those who contribute to the research and development of chitosan-based biomaterials, but will also provide the basis for future research.

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From Chitin to Chitosan

Dayong Teng

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1.1 Chitin

1.1.1 Discovery and Origin

Chitosan usually exists in nature as chitin, which is a natural macromolecular compound, namely, a polysaccharide formed by connecting *N*-acetyl-2-amino-2-deoxy-D-glucoses through β-(1,4) glycoside bonds. The structural formula of chitosan is shown in Figure 1.1. Chitosan is formed when some acetyls are removed from chitin. Usually, products with over 60% deacetylation degree (DD) or that can be dissolved in dilute acid are called chitosan.

In 1811, H. Braconnot, a French professor of natural science history, repeatedly treated mushroom with a warm dilute alkali solution and finally harvested white fibroid residues [1]. He believed that they were celluloses from mushroom and named the residues fungine. In 1823, another French scientist, A. Order, separated the same substance from the elytra of beetles. He named the compound chitin because he thought that the compound was a new cellulose. In 1843, J. L. Lassaigne found that chitin contains nitrogen, which proved that chitin is not a cellulose, but a new compound with fiber quality. In 1878 glucosamine and acetic acid in chitin were identified by G. Ledderhose through hydrolysis, and in 1894 E. Gilson further proved that chitin contains glucosamine. Later research showed that chitin is formed by polymerizing *N*-acetylglucosamine.

In 1859, a Frenchman, C. Rouget, boiled chitin in a concentrated alkali solution for a while and found that the product can dissolve in organic acid after washing it [2]. In 1894, F. Hoppe-Seiler confirmed that the product is deacetylated chitin and therefore named it chitosan.

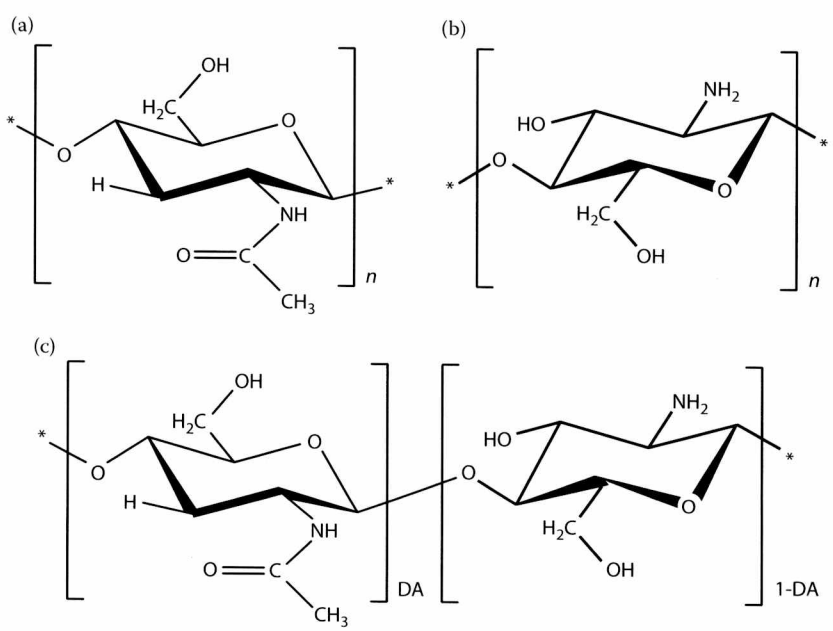

FIGURE 1.1
Chemical structure of (a) chitin and (b) chitosan repeat units. (c) Structure of partially acetylated chitosan, a copolymer characterized by its average degree of acetylation DA.

1.1.2 Existence

As a natural organic compound, cellulose content is the largest on Earth, followed by chitin content. Cellulose is produced from plants and chitin is produced from animals. It is estimated that approximately 10 billion tons of chitin can be biosynthesized in nature each year. Chitin is also a natural nitrogen-containing organic compound with the largest content on Earth, except for protein. Sources of chitin are the following.

1. Arthropods: the primary source is crustacea such as shrimp and crab whose chitin contents are 58–85%, and the secondary source is insects (e.g., locust, butterfly, mosquito, fly, and silkworm chrysalis) whose chitin contents are 20–60%, and myriapods and arachnids.
2. Molluscs include amphineura, gasteropods, scaphopoda, lamellibranch, and cephalopods whose chitin contents are 3–26%.
3. Annelids include archiannelida, chaetopoda, and hirudinea, some of which contain little chitin whereas others contain 20–38% chitin.
4. Protozoans, namely unicellular animals, include mastigophora, sarcodina, sporozoa, and ciliatea, which contain a little chitin.
5. Coelenterates include hydrozoa, scyphozoa, and actinozoa, some of which contain little chitin while others contain 3–30% chitin.
6. Seaweed: the primary source is green algae, which contains just a little chitin.
7. Fungi include ascomycetes, basidiomycetes, and phycomycetes whose chitin contents are from trace to 45% chitin. Only a few fungi do not contain chitin.
8. Others include rigid parts of animal joints, hoofs, and feet and joints of muscles and bones.

All the chitins found in nature exist as complex compounds rather than as separated or alone. In the shells of insects and the exoskeletons of molluscs, chitins combine with proteins to harden the cuticles of insects by cross-linking with polyhydric phenol. In fungi, chitins combine with other polysaccharides such as cellulose [3].

Chitins can be divided into α-, β-, and γ-chitins due to hydrogen bonds. α-Chitin comprises two antiparallel polysaccharide chains. β-Chitin comprises two parallel polysaccharide chains. γ-Chitin comprises three parallel polysaccharide chains, two of which are in the same direction. α-Chitin is the most stable one; thus it has the largest content, and the other two types can transform into α-chitin if conditions permit. Different configurations lead to different functions. α-Chitin can be found in parts with high hardness (e.g., the cuticle of arthropods) and usually combines with shell protein or inorganic compounds. γ-Chitin and β-chitin exist in soft and firm parts. All the three chitins can be found in inkfish: α-chitin forming a thin esophageal epithelium in the stomach, β-chitin forming the skeleton, and γ-chitin forming a thick esophageal epithelium in the stomach [4].

1.1.3 General Situation of Research and Production

Between 1811 (when chitin was first identified) and 1910, there were only 20 research papers on chitin and chitosan in the literature. In the 1930s, the very first patent for industrial preparation of chitosan and a patent for the preparation of chitosan film and chitosan fiber were issued in the United States, which had promoted research on chitin and chitosan. But chitin and chitosan did not attract research attention until the 1970s. In 1977, scientists

began to be attracted by this resource due to the first monograph of Professor Muzzarelli from Cambridge University and the First International Conference on Chitin and Chitosan [5]. The *First International Conference on Chitin and Chitosan* was held in Boston from April 11, 1977 to April 13, 1977, and scientists from the United States, the former Soviet Union, Japan, Norway, Canada, South Africa, Belgium, Britain, Nigeria, India, Italy, and Chile attended this conference. During the conference 47 reports were submitted, which focused on distribution of chitin and chitosan in nature, the separation method, properties, and applications in different fields, especially application of chitin and chitosan in wastewater treatment. This conference was a milestone in the research, development, and applications of chitin and chitosan.

Thereafter, many countries started to provide investments, resources, and labor power for research and industrial applications of chitin and chitosan. During this period, Japan had made great achievements in research and applications [6]. Many of their findings were advanced. Moreover, other countries and regions such as China, Korea, Singapore, and Thailand also had some valuable findings in research. From then on, China began to play an increasingly important role in research in Asia and the world.

In October 1996, the Chinese Chemical Society held the first *China Chemical and Application Symposium on Chitin* in Dalian, opening Chinese chitin/chitosan academic exchanges. In November 1997, the *Chitin Resources Research and Development Seminar* was held in Qingdao. In October 1999, the Chinese Chemical Society held the second *China Chemical and Application Symposium on Chitin* in Wuhan, and during the meeting, the Chinese Chemical Chitin Seminar was formally established. In 2001, the third *China Chemical and Application Symposium on Chitin* was held in Zhejiang Yuhuan County; it was a milestone in chitin/chitosan research. Then, in 2004, the fourth *China Chemical and Application Symposium on Chitin* was held in Guangxi Beihai. In 2006, the fifth *China Chemical and Application Symposium on Chitin* held in Nanjing had a profound influence, and in order to confirm the need for developing and promoting application research, the meeting was renamed the fifth *Chinese Chemical Society Chitin Chemical Biology and Application Technology Symposium*. From the contents of the papers presented at the conference, fundamental research and innovation achievements significantly increased, and the number of young researchers increased significantly, indicating a vigorous development period for Chinese chitin/chitosan research. In June 2006, the Chinese Chemical Society professional committee on chitin, the Chinese Society of Biotechnology professional committee on sugar biotechnology, and the Chinese Society of Oceanography professional committee on marine bioengineering sponsored a chitin and its derivatives conference in Qingdao, and more than 30 experts leading research on chitin/chitosan made a congress report. From the report, research on medicine and biological materials has become a focus, indicating that chitin/chitosan have a wide range of potential applications in nanobiomaterials, bioactive materials, and environmental-friendly functional materials.

Since 1977 when the first *International Conference on Chitin and Chitosan* was held in Boston, another 10 conferences on the same topic have been held. The eighth, ninth, and tenth conferences held in Japan, Canada, and France, respectively, were greatly enhanced in scale, number of attendees, and topics, reflecting that scientists highly value chitin and chitosan. Several monographs on chitin and chitosan were also published abroad [7–9].

Over the last 10 years, chemical modification methods of chitin/chitosan have improved a lot owing to the development of environment-friendly functional materials and subjects merging. The modifications not only helped research on the structure–activity relationship, but also contributed to the development of special functional polymer materials. Chitin and chitosan relevant research and product development are in full swing now.

An upsurge of interest in chitin/chitosan and their derivatives has swept the globe, and thus many countries are putting more efforts into relevant research and development. Since 2000, the number of research papers on chitin/chitosan has been increasing linearly. These usually concentrate on the application of chitin/chitosan and their composites in biomedical fields such as tissue engineering, gene vectors, and drug carriers, which indicates that chitin/chitosan and their derivatives are important in the research and application of biomaterial in the twenty-first century.

Now, in industrial preparation, chitin is formed by removing calcium carbonates and proteins from waste shrimp shells and crab shells from aquatic product factories by steeping them in acid and alkali solutions. This technique has many inherent shortcomings; for instance, the raw materials are hard to collect, preserve, and transport owing to limitations of location and season; resources of raw material are very different; the quality of products can hardly be controlled; and large amounts of calcium carbonate in the shells make extraction of chitin difficult, which increases cost and generates plenty of wastewater [10]. Therefore, new chitin resources have drawn a lot of attention: for example, various insects that are abundant in nature such as pine moth [11], myiasis [12], silkworm chrysalis [13], and cicada slough [14].

1.2 Deacetylation

Chitosan can be harvested by removing acetyls from chitin, but to eliminate all acetyls is not easy. Chitosan can be made by a chemical method or an enzyme method. Chitosans available in the market are formed by removing acetyls from chitin through strong base hydrolysis. The equation is shown in Figure 1.2.

1.2.1 Chemical Method

The chemical method for preparing chitosan includes the alkali fusion method, the concentrated alkali solution method, the alkali catalysis method, and the hydrazine hydrate method. The main performance indexes of chitosan are DD and relative molecular weight or viscosity. To date, quite a few researchers have studied chitosan preparation and have made significant achievements [15,16]. Research findings on the extraction process of chitosan from shrimp and crab shells are shown in Figure 1.3.

The alkali fusion method is used in the early period, comprising the following steps: fusing chitin and solid potassium hydroxide directly in a nickel crucible, melting them at

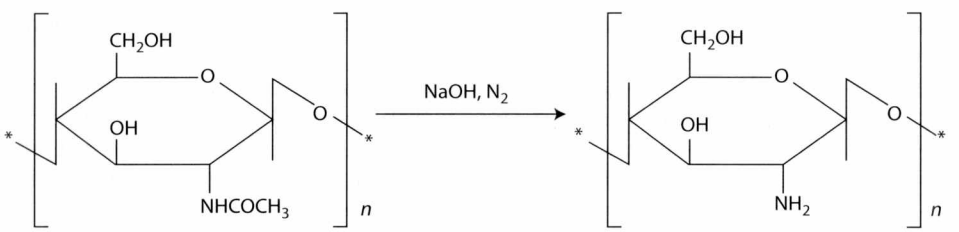

FIGURE 1.2
Preparation of chitosan by base hydrolysis of chitin.

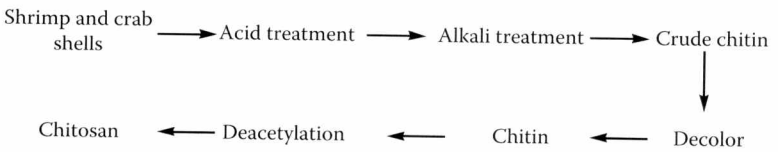

FIGURE 1.3
Preparation process of chitosan.

180°C for 30 min under nitrogen protection, transferring the mixture into ethanol solvent to form gelatinous depositions, washing the depositions with water to neutral pH in order to form rough chitosan, dissolving the rough product in 5% formic acid, neutralizing the solution by using diluted NaOH to form deposits, filtering the mixture, washing the deposits to neutral, and forming refined chitosan by repeating the said steps. The product undergoes serious main chain degradation and hence the relative molecular weight is small; also, the method is complicated. As a result, it was abandoned. The concentrated alkali solution method is the most popular one, in which raw materials react with 40–50% NaOH solution at 100–130°C for 0.5–6 h and chitosans with different DDs are generated. The concentration of alkali solution, reaction temperature, reaction time, and shape of solid chitin are closely related to DD.

The concentration of alkali solution, reaction temperature, and reaction time are the main factors affecting chitosan performance (viscosity and DD). Orthogonal experiments show that all three factors can influence the performance to varying degrees, and the most important one is NaOH concentration [17]. Considering the main quality standards of chitosan, which are viscosity and DD, the preparation method includes the following steps: mixing milled chitin with 45–50% NaOH in a weight/volume ratio of 1:10, reacting the mixture at about 90°C for 8–10 h, controlling the temperature carefully and stirring the mixture continuously during the reaction, washing the product with water to neutral, and drying the product to form white chitosan powder. To accelerate deacetylation, discontinuous water washing can be used.

In homogeneous phase, when DD is about 50%, chitosan will have good water solubility. However, when the reaction occurs in heterogeneous phase, the product is water insoluble despite 50% DD [18]. Analysis of chemical structure proves that acetamino and amino irregularly distribute in chains of the water-soluble chitosan with 50% DD, breaking molecular orderliness; that is why the product is water soluble. The water-soluble chitosan has high solubility and is alkali soluble, so that the modification reaction can be carried out in alkali conditions, expanding the range of chitosan research and application [19]. Although deacetylation in homogeneous phase can generate water-soluble chitosan, the reaction must occur in concentrated alkali solution and a large amount of solvent is needed for desalting in the late stage; therefore, this method is not applicable for industrial use.

The concentrated alkali solution method requires largely excess NaOH, which is a waste product. Organic solvent can strongly permeate chitin and help alkali enter the chitin as a diluting medium; thus organic solvent will reduce the amount of alkali while chitosan with high DD can still be formed. A batch process can form chitosan with high DD and high quality. When the reaction temperature is 60°C, acetone will produce higher DD and relative molecular weight than ethanol. However, the product is yellowish and difficult to wash when the solvent is acetone. When the reaction temperature is 80°C, water is worse than ethanol as reaction medium because it causes low DD, unsatisfactory color, and makes

the product hard to wash. Therefore, ethanol is the best medium for deacetylation. Ethanol with a certain polarity and penetrability can efficiently penetrate into chitosan to increase reaction efficiency. When the reaction occurs at 80°C with ethanol as the medium for 3 h, DD can reach 90% if the weight ratio of chitosan to NaOH to ethanol is 1:3:16, while DD is 80% in the traditional method [20].

Microwave radiation heating greatly reduces alkali treatment time compared with the traditional heating technique for preparing chitosan, and makes chitosan have high DD and solubility. In 1979, Peniston tried to prepare chitosan by treating chitin with microwave radiation in the normal alkali solution method for the first time [21]. Chitosan with 85% DD can be formed by the normal alkali solution method by reacting in 50% NaOH solution at 100°C for 10 h. When microwave is used, only 80°C and 18 min are enough for chitosan with over 80% DD. The semi-dry microwave method can also be applied for chitosan preparation, comprising the following steps: uniformly mixing a concentrated alkali solution with chitin, which is milled into a certain granularity in advance to form a paste, deacetylating in a microwave oven, washing the product using hot water to neutral, steeping the product in methanol, and drying the product in vacuum to form white or yellow grains [22].

The microwave radiation greatly shortens deacetylation time and lowers energy consumption. But radiation also seriously breaks the chitin chain, making the product low in relative molecular weight. Hence the method is particularly suitable for making chitosan with high DD and low relative molecular weight. Microwave treatment increases the reactivity of chitin and the reaction rate of deacetylation; hence the reaction time is shortened and the alkali amount is reduced. It is a good way of saving material and lowering energy consumption, so that the product cost of chitosan is saved. The industrial microwave reactor will definitely bring remarkable benefits if it is developed.

The alkali solution catalysis method is suitable for chitosan with high DD and high relative molecular weight. This method uses thiophenol and dimethylsulfoxide in addition to NaOH. The thiophenol is transformed into sodium thiophenol with deoxidizing and catalytic functions in NaOH solution. Therefore, the reaction is accelerated and chain breakage is prevented. The reaction medium is an alcohol–water solution of NaOH. The phase transfer catalyst is cheap and harmless polyethylene glycol with good human compatibility (no need to remove the catalyst after reaction). The reaction condition is moderate and with high DD, which can be obtained when concentration of NaOH is 35%, reaction temperature is 90°C, reaction time is 3 h, and concentration of the phase transfer catalyst is 5%. This technique can remove protein and prevent the degradation of chitin when alkali concentration is low. It lowers acid and alkali consumption and shortens the production period [23]. But it can only be used for preparing small amounts of samples in the laboratory.

1.2.2 Enzyme Method

Chitin deacetylase can hydrolyze acetyls of chitin, and so it may replace the hot concentrated alkali method for producing high-quality chitosan. Chitin deacetylase was first found in *Mucor rouxii* of zygomycetes in 1974 [24]. Electrophoretically pure chitin deacetylase from *Mucor rouxii* with specific activity 13.33 U/mg can be formed by immune affinity chromatography, and the yield is 29.1% [25]. Chitin deacetylases from different sources differ with respect to relative molecular weight, isoelectric point, optimum pH, inhibitor, and distribution, leading to different physiological functions. The reaction mechanism of the chitin deacetylase from *Mucor rouxii* is multipoint attack mode; specifically, the enzyme systematically hydrolyzes acetyls from the nonreducing end of the binding site after it binds to a

substrate chain, and then leaves the substrate and binds to another one. There is no binding tendency between the enzyme and molecular sequence of the substrate [26]. Strains that produce a large amount of extracellular deacetylase with high activity are very valuable in the production of chitin deacetylase and the production of chitosan by the catalytic method.

The chitin deacetylase method could replace the hot concentrated alkali method because it prevents serious environmental pollution, lowers energy consumption, and solves the problem that product treated with hot concentrated alkali has uneven DD and low relative molecular weight. The product formed by the enzyme method can be used for producing new functional materials. Nevertheless, there are still some problems such as low yields of deacetylase-producing strains and low enzyme activity. Moreover, natural chitins are crystals, not a good substrate for deacetylase. Hence, many preparations still need to be carried out before the chitin deacetylase method can be used in the industrial production of chitosan.

The microorganism culture method is another hotspot of chitosan research, which removes acetyls by catalyzing the substrate with enzymes produced by microorganisms. From the 1980s, Japan and the United States began to study chitosan production by microbial fermentation [27–29], followed by China from the early 1990s. Currently, the research concentrates on breeding of the strain and optimization of the culture medium. The chitosan formed by this method is similar to the chitosan from shelled animals in terms of DD and relative molecular weight, while its metal ion adsorption capacity is much higher. So the product is particularly suitable for treating heavy-metal-ion-containing wastewater. The antibacterial ability of the food preservative made of the product is 1–2 times that of the food preservative made of chitosan from shelled animals. It can be seen that the microorganism culture method has good prospects.

1.3 Control of Quality

1.3.1 Deacetylation Degree

The DD of chitosan, namely the content of free amino in chitosan chains, is a technical index of great importance. The DD of chitosan directly relates to solubility in diluted acid, viscosity, ion exchange ability, flocculability, reaction capacity with amino, and other aspects.

DD can be defined as the ratio of residues without acetyls to all residues of chitosan. Quite a few methods can be used for measuring DD, such as alkalimetry (acid–base titration [30–33], electrolytic titration [34–35], and hydrobromide titration [36]), infrared spectroscopy [37–41], refractive index [42], colloid titration [43,44], thermal analysis [45], gas chromatography [46], ultimate analysis [46], ultraviolet (UV) spectrometry [44,45], and trinitrophenol spectrophotometry [37]. The most common method is acid–base titration, followed by infrared spectroscopy and electrolytic titration.

Acid–base titration is the simplest method with good repetitiveness for measuring the content of free amino in chitosan, and does not require special instruments. This method is particularly suitable for monitoring weight during production. The mechanism is that the alkali free amino in chitosan can be protonated by acid quantification to form a chitosan colloid solution, then the dissociative hydrogen ions can be titrated by alkali, and acid combined by free amino can be figured out by the difference of acid for dissolving chitosan and alkali for titrating.

Precautions in acid–base titration are as follows: (1) To prevent the error caused by hydrochloric acid degrading the main chain of chitosan, the sample should be dissolved at room temperature and not at high temperature. (2) The higher the DD, the larger the solubility of the sample, and vice versa. Hence some samples need to be treated overnight. (3) The deacetylation is uneven, usually resulting in incomplete dissolution. If data from three measurements are very different, the sample should be measured again. (4) The sample must be neutral, or the result may be incorrect. The sample that is not neutral should be washed to neutral or the data should be corrected. (5) The influence of oxygen in the atmosphere can be ignored, and hence nitrogen protection is not necessary. (6) Obvious agglutination lowering the measured values should be prevented. (7) The color of the colloid solution with large viscosity changes slowly when the titration end point is near, and hence the operator should pay attention to the rate of titration.

DD can be measured by the absorption peak of characteristic groups in the infrared spectrum of chitosan [47]. In this method, it is not necessary to dissolve chitosan by solvent or solution, which means that the infrared spectrum can be directly obtained by using dry powder. By using a series of samples with known DDs, a standard curve can be plotted by using the absorption peak ratio of special bands such as amide I or amide II to a certain band. The DD of the tested sample can be determined by using the curve. Compared with acid–base titration, the error is larger in this method. But the infrared spectrum is more convenient and samples used in the method can be recycled. Dryness is significant to the repeatability of experimental data. Generally, the amide I peak is hardly affected by water while the amide II peak is not; hence the sample must be dried carefully. Furthermore, the sample must be ground into very fine powder, or the absorption peak will not be sharp enough, making the determination of peak height inaccurate.

Electrolytic titration has the same mechanism as acid–base titration, but is different from the end point determination method, which means that electrolytic titration uses a potential curve while acid–base titration uses a single indicator or indicator mixture [34].

Electrolytic titration comprises the following steps: dissolving chitosan in a standard hydrochloric acid solution in a small beaker, measuring the standard NaOH titration process by a potentiometric titrimeter, recording pH when 0.25–0.5 mL of NaOH is used, recording pH more frequently when the end point is near, plotting a pH–V curve by using pH as the vertical coordinate and the volume of NaOH as the horizontal ordinate, finding the volume of NaOH corresponding to the equivalent point, and calculating DD by the given formula.

There are still some disadvantages: (1) Chitosan that separates out before and after the end point may cover the electrode film, influencing the measurement of pH. (2) It is different to find out the equivalent point on the titration curve, which is usually shaped as S, causing personal error in the determination of the end point.

1.3.2 Molecular Weight

Relative molecular weights of chitin and chitosan can be measured by gel permeation chromatography [48], steam osmotic pressure method, membrane osmometry, end group method [49], viscosity measurement, light scattering method, and coupled light scattering–gel permeation chromatography. Gel permeation chromatography is applied for measuring weight-average relative molecular weight and number-average relative molecular weight. The light scattering method is applied for measuring weight-average relative molecular weight. The viscosity measurement is applied for measuring viscosity-average relative molecular weight.