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Journal of Analytical & Pharmaceutical Research

Mini Review

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Analysis of gelatin using various separation and detection technologies

Abstract

It has been increasingly important for pharmaceutical scientists and engineers to gain a better understanding of gelatin raw materials, intra/intermolecular interactions, conformational changes and physicochemical properties of gelatin-based softgel capsule, hard gelatin capsule, and gummy product development and manufacturing processes. Such comprehensive data often needs utilization of a combination of systematic experimental designs and execution. In this paper, we reviewed a series of research articles focused on study of fresh and aged gelatin raw materials and softgel products using from conventional size exclusion chromatography (SEC) coupled with various detection systems to field-flow fractionation (FFF) and asymmetrical flow field-flow fraction coupled with multi-angle light scattering detection systems (AFIFFF-MALS). Conventional SEC is considered to be suitable to analyze lower molecular weight gelatin molecules and polypeptides when low molecular weight standards were used. The solvent systems used range from near physiological conditions to very harsh solvents including high concentrations of salts and/or high concentrations of strong surfactants. The solvent systems with high salts or high surfactant created complete denaturation or elimination of higher orders of protein structure of gelatin molecules. This typically would achieve good separations and acquire accurate molecular weight information. However, the detailed information of true conformational structures and intra /intermolecular interactions of gelatin molecules could not be obtained. When the solvent systems that are close to physiological conditions (i.e. pH, ionic strength, salts, and buffering agents) were employed, the information on true molecular interactions, molecular weight, conversion, and solution conformation of gelatin molecules could be obtained. Nonetheless, some data indicated that abnormal size exclusion separation and conformational changes occurred because the undesirable interactions between gelatin molecules and SEC column packing materials might not be completely eliminated. In addition, SEC normally has poor resolution in the ultra-high molecular weight region (i.e. greater than approximately 1 million Da) because of the total size exclusion limit of packed columns. The satisfactory separation of some ultra-high molecular weight components including microgels and particulates could not be achieved. In contrast, FFF-MALS or AFIFFF-MALS is considered to be an alternate method since it does not have the size exclusion limit and free of column packing materials. It is suitable for analyzing ultra-high molecular weight components such as microgels and particulates in gelatin materials using close to physiological conditions. Conversely, the limitation of AFIFFF-MALS is that the system is not effective in analyzing low molecular weight components (i.e. less than 5k Da).

Keywords: gelatin, molecular weight, molecular conformation, SEC, FFF, AFIFFF, MALS, ultra-high molecular weight components, exclusion limit

Volume 7 Issue 6 - 2018

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Received: December 01, 2018 | **Published:** December 26, 2018

Abbreviations: SEC, size exclusion chromatography; FFF, field-flow fractionation; AFIFFF-MALS, asymmetrical flow field-flow fraction coupled with multi-angle light scattering detection systems; APIs, active pharmaceutical ingredients; CoA, certificate of analysis; GPC, gel permeation chromatography; PD, polydispersity index; SEC-TD, SEC with triple detection; SEC-MALS, SEC with multi angle light scattering; AFIFFF, asymmetric flow field-flow fractionation

Introduction

Gelatin is a mixture of water soluble proteins and polypeptides which are products of chemical degradation and partial hydrolysis of fibrous collagen from various animal sources. Collagen is the primary fibrous protein in animal (i.e. fish, cow, chicken, and pig) bones, cartilage and skins. The use of gelatin plays a very important role in pharmaceutical, nutraceutical, food, biochemical, and chemical industries. Gelatin has been used as a gelling agent, binder, emulsifier, adhesive, and processing aid. It is particularly crucial in pharmaceutical and nutraceutical industries as a means of active pharmaceutical ingredient protection, taste masking, and drug delivery systems. Dosage forms such as gelatin-based hard gel capsules, soft gel capsules, and gummies use considerable amount of gelatin raw materials, plasticizers and water. Gelatin has been utilized widely for coating of tablets and beadlets to protect active pharmaceutical ingredients (APIs). Additionally gelatin is also used in the preparation of granulation and as binder in various solid dosage forms.

The U.S. Pharmacopoeia/National Formulary defines gelatin as a product obtained by the partial hydrolysis of collagen derived from the skin, white connective tissue and bones of animals.[1] Gelatin derived from an acid-treated process is known as Type A, and gelatin derived from an alkali-treated process is known as Type B. Type A is generally derived from pigskins and fish skins, while Type B is generally derived from bovine bone and hide.

In transforming fibrous collagen into gelatin, the effects of acid or base hydrolysis, enzyme degradation as well as thermal stress and the time treated/ages of the fibrous collagen create diverse types of collagen fragments. These fragments consist of three polypeptide chains (α chains) which are twisted into left-handed helixes. The helical structures of polypeptide chains are stabilized primarily by hydrogen bonds. The high molecular weight chains and/or fragments were created when less hydrolysis took place. The lower molecular weight fragments or polypeptides were generally produced through a series of hydrolysis steps at various peptide bonds. These processes give rise to a mixture comprising different molecular weights, molecular weight distributions and molecular conformations of gelatin. Derived from the compact triple helix collagen, gelatin chains possess thermally reversible gelling properties in which they can undergo conformational changes between random coil and helix structure at different environmental conditions.[1]

The certificate of analysis (CoA) of gelatin raw materials from suppliers does not provide information on molecular weight or molecular weight distribution of the material, and only lists gel strength (or Bloom strength) and solution viscosity values at a concentration of 6.67% at 60°C. Although the solution viscosity generally correlates with viscosity-average molecular weight based on Mark-Houwink equation in dilute solutions, this does not provide any information on the molecular weight distribution of the complex gelatin raw material. Since gelatin is known to easily undergo conformational changes between coil and helix, crosslinking as well as thermally degrade at high temperatures over time, it is critical for us to know exactly how the environmental conditions affect the product properties and performances. Furthermore, gelatin typically has a very broad molecular weight distribution, we believe that the detailed determination of molecular weights, molecular weight distributions, solution conformations, and intra/intermolecular interactions of gelatin in different matrices would help better understand structure/property relationships as well as improve processing conditions and predict the product performances at the molecular level. Quality control of molecular weight and molecular weight distribution of gelatin is critical for its broad applications.[2]

Discussion

Early work showed that the molecular weight distributions of gelatins could be determined by gel filtration and ultracentrifugation fractionation, with measurement by the concentration detection (UV-vis and differential refractive index of dRI) and light scattering techniques.[3] The molecular weight ranges from oligo-peptides of 100 Da to large fragments or microgels of greater than $1x10^6$ Da was obtained. Conventional SEC coupled with a dRI and/or UV-vis is considered to be suitable to analyze lower molecular weight gelatin molecules or polypeptides when properly low molecular weight standards were used. The molecular weight distribution determined by modern analytical methods including gel permeation chromatography (GPC) or SEC has been reported to range roughly from 10,000Da to 400,000Da for both type A and type B of gelatin.[1,4] Moreover, certain amount components with ultra-high molecular weight (greater than one-million Da) which were labeled as 'microgels' have been reported.[5–7]

Table 1 summarizes the experimental conditions in recently published papers on separation and characterization of gelatins and water soluble collagen. As shown in the table, various buffered solutions with different concentrations of salts and/or surfactants were used for the gelatin separation and analysis. It is worth noting that the dn/dc values from different literature ranged from 0.163 to 0.190. The dn/dc value is very important for the MALS to precisely calculate the absolute molecular weight and molecular conformation. This might have suggested some contradictions/deviations in absolute molecular weights and molecular conformation of gelatin samples determined from different research laboratories. Regarding the solvent systems used, there are two different types of mobile phases, the first type[2,4] utilized very harsh solvent systems (strong surfactant 1% SDS and strong salt 1M $CaCl_2$) which completely destroyed the higher order structure and denatured the gelatin molecules and soluble collagen materials. Under those conditions, distinct or aggregate peaks were observed for the gelatin as well as water soluble collagen samples. However, these observations do not resemble the natural states of gelatin molecules in the physiological environment and real pharmaceutical applications and manufacturing processes. Furthermore, these aggressive solvent systems are very harsh and can damage the seals and injection ports of the SEC system. The second type used more gentle salts and buffering agents.[5,7–9] Under these conditions, the gelatin materials are analyzed were in their more natural and pharmaceutical relevant states.

Table 1 Summary of SEC and AFIFFF conditions used in recently published literature on separation and characterization of gelatins and/or water soluble collagen

Columns/AFIFFF plate Membranes	Column Temperature	Mobile Phase	dn/dc Reported	Reference
2 x TSK-gel PW30 + TSK-gel PW50	50°C	0.1M Na_2SO_4, 0.01M NaH_2PO_4, 1%SDS, pH 5.3	N/A	Wu C[2]
Ultrahydrogel Linear 300x7.8mm	Ambient	0.15M NaCl, 0.1M phosphate, pH 6.8	N/A	Farrugia CA[5]
TSK-gel 6000 PW + TSK-gel 3000 PW	40°C,50°C, 60°C,70°C, 80°C	0.125M $LiNO_3$ or $NaNO_3$, 0.01M K phosphate, pH 6.7	0.163	Tromp RH[7]
Superose 6 HR 10/30	Ambient	1M $CaCl_2$, 0.05M Tris, pH 7.5	0.190	Meyer M[4]
Ultrafiltration membrane of regenerated cellulose with 5k Da cut-off	N/A	2mM Na phosphate, 14mM NaCl, pH 6.0	0.164	Rbii K[8,10]
3 X Ultrahydrogel Linear 300x7.8mm	37°C	0.154M NaCl, 20mM Na Phosphate, 5mM TEA, 5% ACN, pH 7.0	0.175	Crawshaw B[9]

Citation: Herrick DZ, Maziarz EP, Liu XM. Analysis of gelatin using various separation and detection technologies. *J Anal Pharm Res.* 2018;7(6):669–672. DOI: 10.15406/japlr.2018.07.00299

The polydispersity index (PD), which measures the broadness of a molecular weight distribution of a polymer, is the quotient of weight average molecular weight (M_w) and number average molecular weight (M_n). Gelatin normally has a broad molecular weight distribution with a PD greater than 4[5,8,10] with an exception of over-processed gelatin materials used for special softgel applications including crosslinking prevention and fast release.[9,11,12] The physical properties of gelatin are greatly dependent on its molecular weight and molecular weight distribution. Gel strength is mainly dependent on the population at approximately 100,000Da and higher molecular weight, while the distribution at 200,000 Da to 400,000Da has an important effect on viscosity. The viscosity of a gelatin solution correlates relatively well with the proportion of high molecular weight components in dilute solutions.[1]

Currently there are few SEC or GPC publications from pharmaceutical and nutraceutical industries in the scope of detailed gelatin characterization. Crawshaw B et al.[9] developed effective aqueous SEC with triple detection (SEC-TD) and aqueous SEC with multi angle light scattering (SEC-MALS) methods to characterize type A and B gelatin raw materials under near-physiological conditions. The SEC-TD provided absolute molecular weight values, intrinsic viscosity, viscosity, hydrodynamic radius and concentration at each elution moment by using a differential refractometer, a differential viscometer, and a two-angle light scattering detector. The SEC-MALS yielded absolute molecular weight and root mean square radius (also known as radius of gyration), and concentration at each elution moment.[9] Two possible shortcomings from the SEC experiments[4,5,9,13] are the poor resolution in the ultra-high molecular weight region and the component carry-over. The poor resolution in the ultra-high molecular weight region is the inherent property and total size exclusion limits of the packed SEC column technology. There exists an upper limit for a SEC column to effectively separate the ultra-high molecular weight gel and components. The carry-over issues can be caused by undesirable interactions between the gelatin molecules and column packing materials. Two approaches can be used to minimize or eliminate these undesirable interactions: First, the column packing materials with minimal or free of reactive functionality including residual carboxyl and amine groups should be utilized. This approach can effectively minimize the possibly undesirable interactions between the column packing materials and gelatin molecules even no high concentration of salts or surfactants is used in the mobile phase. Second, one can use stronger solvent systems (i.e. strong surfactants and high concentration of salts) as well as end-capping reagents to minimize the interactions between gelatin molecules and the column packing materials.

More recently, field flow fractionation (FFF) and asymmetric flow field-flow fractionation (AFIFFF) combined with MALS were used to characterize gelatin and other biomaterials.[8,10,14–16] Unlike the SEC/GPC, FFF or AFIFFF coupled with MALS does not have the restrictions of high shear stress and total exclusion limit, and therefore it can be ideal in characterizing ultra-high molecular weight components. Rbii et al.[8,10] demonstrated the advantage of AFIFFF-MALS in characterizing high molecular weight gelatin aggregates due to aging and thermal treatment, also made possible by using gentle experimental conditions, which both would not be achievable with SEC due to abrasive shear stress, trapping in and interactions with the column packing material. One of the main shortcomings of FFF-MALS/AFIFFF-MALS is that it is not effective in analyzing low molecular weight components of gelatin (i.e. less than 5k Da). Thus, SEC with different detection systems and FFF-MALS/AFIFFF-MALS complement each other very well.

Conclusion

Accurately and precisely characterizing gelatin raw materials and gelatin-based products has great importance in pharmaceutical, foods, and consumer health industries. The separation condition selection is critical to obtain reliable and complete information on molecular weight distributions, intra/intermolecular structures and conformations of gelatin materials throughout the product development processes. Many experimental conditions have been reported in the literature, but some of them have contradictions/deviations and need in-depth evaluation. The information obtained from proper conventional SEC, SEC-MALS, and FFF/AFIFFF-MALS allows researchers to: accurately characterize the molecular weight, molecular weight distribution, conformation, and solution properties of gelatin samples. These can be potentially powerful tools for qualifying gelatin raw materials, evaluating manufacturing processes, assessing product performance and stability at molecular structural level. Due to the extremely broad molecular weight distributions of gelatin materials, conventional SEC complements well to FFF/AFIFFF-MALS. Combing conventional SEC, SEC-MALS and FFF/AFIFFF-MALS can be powerful to troubleshoot production problems and develop new gelatin-based products and processes.

Acknowledgments

None.

Conflicts of interest

The author declares that there is no conflicts of interest.

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Citation: Herrick DZ, Maziarz EP, Liu XM. Analysis of gelatin using various separation and detection technologies. *J Anal Pharm Res*. 2018;7(6):669–672.
DOI: 10.15406/japlr.2018.07.00299