Fabrication of Solid Collagen Nanoparticles Using Electrospray Deposition

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Collagen is a promising biomaterial for drug delivery due to advantages including high biocompatibility and biodegradable property. However, transforming collagen into solid nanoparticles is difficult, although the solid dosage form is advantageous for some administration routes including pulmonary and oral drug delivery. In this study, collagen solid nanoparticles are prepared in one-step using electrospray deposition under ambient temperature and pressure conditions. Although collagen molecules formed micron-sized aggregates in acetic acid solutions spontaneously, electrospraying the collagen solutions resulted in formation of nanofibers. Solid nanoparticles were obtained by increasing conductivity of the solution and/or inducing structural perturbation of the collagen molecules using salts. The ability of solid collagen particles as a drug carrier was demonstrated by incorporating theophylline as a model drug using a coaxial spray technique. Release of theophylline was controlled by cross-linking collagen molecules. Electrospray deposition was proved to be a powerful method for producing solid collagen nanoparticles for drug delivery.

Key words: nanoparticle; collagen; controlled release; cross-linking; electrospray deposition

Nano-scale drug-delivery systems possess major advantages because nano-scaled materials can exhibit distinctive physical, electrical, mechanical and optical properties that differ from those observed in macroscopic and atomic realms. Nano-scale drug delivery systems can be manufactured using both physical and biological methods, for various applications including oral, injectable, inhalable, and transdermal drug delivery. Notably, attention on nanoparticulate oral drug delivery systems is increasing because of the need to overcome the physicochemical problems of new chemical entities, since nano-sizing can enhance the dissolution rate of poorly soluble drugs. In addition, penetration and retention of drug molecules in a mucous layer may be improved using nanoparticles. In many cases, polymeric materials are playing important roles for designing nanoparticulate drug delivery systems.

Many efforts are ongoing to utilize the pulmonary route for systemic drug delivery because of the advantages compared to oral delivery, such as rapid action, avoidance of enzymatic degradation, and easier penetration of large molecules across biological membranes. For pulmonary drug delivery, particulate solid formulations can promote stabilization and administration of a large amount of drug compared to other pulmonary delivery technologies including use of metered dose inhalers and nebulizers. Particle size is widely recognized as one of the most important factors in determining the deposition site of the drug in the lung. There are several deposition models available. The most famous one is the International Commission on Radio Protection (ICRP) model, which was developed by assembling experimental observations with model calculations that considered complicated lung morphology. According to this model, particles smaller than 100 nm most effectively reach the alveoli region. However, this size of particles is almost impossible to manufacture using current formulation technology. Moreover, even if such a formulation can be developed, it will be difficult to prevent aggregation during storage. Thus, micron-sized particles are regarded as the best option for pulmonary drug delivery.

Only limited number of excipients is available for pulmonary drug delivery, which means that the degrees of freedom for formulation design are very narrow. Collagen is a good candidate for inhalation use due to its fine biocompatibility and low antigenicity. However, the preparation of collagen particles usually involves multiple complicated steps, which are not practical for industrial productions. In addition, particles are obtained as suspensions, which are likely to cause aggregation after solvent removal. Electrospray deposition (ESD) is a promising method for preparing pharmaceutical solid particles. ESD has many advantages including the use of mild conditions as well as simplicity of the preparation procedure. Thus, ESD is frequently applied for producing collagen fibrous materials. This technology is referred to as electrosprinning, when fibrous materials are prepared.) Collagen must be fabricated into a particulate form for use as a pulmonary drug delivery carrier, although the electrospraying process leads to formation of fibers rather than particles when collagen is used. In addition, the solvent selection for this process appears to be very important, because an inadequate solvent may cause denaturation. Although the solvent selection for this process appears to be very important, because an inadequate solvent may cause denaturation. Although 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) or 2,2,2-trifluoroethanol (TFE) has been reported repeatedly to yield collagen nano-fibers, these solvents are known to cause denaturation that alters the characteristics of collagen. Therefore, a need exists for an alternate solvent that provide the appropriate environment for collagen. Moreover, it is favourable to use solvents that are generally regarded as safe. This study describes the attempt to prepare collagen solid nanoparticles using aqueous salt solutions, which could be used for various purposes including as a carrier for pulmonary drug delivery. Moreover, theophylline was loaded in the collagen particles using a coaxial spray.
nozzle to achieve controlled release of the drug. Preparation of “solid” collagen nanoparticles should widen its application as a drug delivery vehicle significantly.

Experimental

Materials Type I Collagen and theophylline (THP) were purchased from Sigma-Aldrich (C9879, St. Louis, MO, U.S.A.) and Tokyo Kasei (Tokyo, Japan), respectively. Acetic acid was supplied from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium chloride (NaCl) and calcium chloride (CaCl₂) were obtained from Nacalai Tesque (Kyoto, Japan). All chemicals were used without further purification.

Preparation and Characterization of Collagen Solutions

A solution of collagen was prepared at a concentration of 1 w/v% in 50–90 v/v% acetic acid solution. Salts such as 0.25, 0.5, and 1 w/v% NaCl and 0.25 w/v% CaCl₂ were also added to control the solution properties. All measurements for the solutions were done after incubation at 25°C for 24 h.

Solution conductivity was measured using a conductivity cell attached to an Orion benchtop multimeter (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at ambient temperature. The kinematic viscosity of the solution was measured using an Ubbelohde viscometer (Kusano Science, Tokyo, Japan) in a thermostated bath controlled at 25°C. The absolute viscosity was calculated by multiplying it by density, which was determined by weight of a 5 mL solution in a volumetric flask.

Circular dichroism (CD) spectra were recorded at 25°C using a Jasco J-820 spectropolarimeter (JASCO Corp., Tokyo, Japan). A scan speed of 20 nm/min was used with an average of three scans per sample. A slit width of 1 nm and a time constant of 1 s were used. A 1-mm cell was used for the experiments. The CD spectra of the samples were obtained after subtracting the reference spectrum, which was obtained using acetic acid solutions. The data was analyzed using K2D2 software, which is accessible at http://www.ogie.ca/projects/k2d2/, for estimating secondary structure content (alpha-helix and beta-strand) of collagen.

The apparent size and zeta potential of collagen aggregates in the solutions were determined by a dynamic light scattering (DLS) instrument (Delsa Nano C particle analyser, Beckman Coulter, Brea, CA, U.S.A.) with a He–Ne laser (632.8 nm, 35 mW) as the light source. All measurements were repeated three times. Mean aggregate size was determined by Cumulant analysis. Results for the particle size analysis are presented in “Supplementary content.”

Preparation of Collagen Membranes

Transparent collagen membranes were prepared to investigate structural properties of the dissolved collagen before electrospraying. The collagen solution (1 w/v%) with 50–80 v/v% acetic acid was placed on flat polyethylene plates, followed by air-blowing over the surface to obtain films. Since collagen does not adhere to polyethylene, the dried films could easily be taken off the plates. Membrane samples were also prepared by introducing 0.25 w/v% NaCl and CaCl₂ to the collagen solution.

Electrospray Deposition (ESD) of Collagen Nanoparticles

Collagen solutions (1 w/v%) were supplied by a syringe pump to the grounded nozzle for spraying. Aluminum foil was placed perpendicular to the nozzle as a deposition target, to which a high negative voltage was applied. Solutions were dispersed into fine droplets by an electrical field generated between the nozzle and target. The solvent evaporated before reaching the target, where the solid particles collected. All preparations were carried out in an acrylic chamber at room temperature. The humidity was controlled to be lower than 20% RH by flowing nitrogen gas. Processing parameters were applied voltage, −25 kV; nozzle diameter, 0.65 mm; flow rate of the feed solution, 0.2 mL/min; distance between the nozzle and target, 10 cm. A schematic representation of the instruments can be found in our previous paper.

Characterization of Collagen Membranes and Nanoparticles

The morphologies of the collagen nanoparticles were observed under a scanning electron microscope (SEM) at an accelerating voltage of 10 or 15 kV. Prior to scanning under the SEM, the samples were sputtered-coated with 30 nm platinum (E-1030 ion sputter, Hitachi, Tokyo, Japan). Particle size was determined through the analysis of SEM pictures using Mac View ver. 4.0 software (Mountech, Tokyo, Japan). Three hundred particles were selected randomly from the image to obtain the Heywood diameter. Below presented is volume-mean diameter.

Fourier-transform infrared (FT-IR) and X-ray diffraction (XRD) studies were carried out on collagen membranes and nanoparticles after the spraying process as described in “Supplemental information.”

Encapsulation of THP Using Coaxial ESD

THP was employed as a model drug for entrapment in the collagen particles. However, THP was not entrapped in the particles efficiently through ESD with a single nozzle as shown later. Thus, a coaxial nozzle was used for preparing the collagen/THP particles. Collagen and THP were dissolved in 50 v/v% acetic acid that contained 0.25 wt% CaCl₂, at a concentration of 10 w/v% and 2 w/v%, respectively, for supply by the syringe pumps to the coaxial nozzle. The THP and collagen solutions were used as inner and outer solutions at the flow rates of 0.2 mL/h and 0.5 mL/h, respectively. A high positive voltage of 12.5 kV and a negative voltage at −12.5 kV were applied to the nozzle and the target, respectively. A steel plate was placed perpendicular to the nozzle as a deposition target. The inner and outer nozzle diameters were 0.4 and 0.8 mm, respectively. The distance between the nozzle and the target was 15 cm. The electrospray was conducted in an acrylic chamber at ambient temperature. The humidity was controlled at lower than 20% RH by flowing nitrogen gas.

Cross-Linking of Collagen//THP Particles

Collagen/THP particles were cross-linked with gaseous glutaraldehyde. Particles were placed in a container inside a desiccator that contained, in the lower part, 5 mL of a 10 w/v% GTA aqueous solution. The container was placed in the dark in an oven at 37°C for 18 h.

Dissolution Study

The dissolution study (n=2) was conducted for collagen/THP particles using pH 7 phosphate buffer at ambient temperature. Ground formulations (1.5 mg THP equivalent) were dispersed in 100 mL of medium under stirring. Aliquots were taken at fixed time intervals, and then filtered using a syringe filter with a pore size of 0.45 pm. Quantification of THP was performed by HPLC with a Cosmosil SC18-AR II column (150 mm × 5.0 mm i.d., Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min. The mobile phase consisted of a mixture of acetonitrile and water at a ratio of 892 (v/v). Injection volume and the detection wavelength were 2 μL and 270 nm, respectively.
Results and Discussion

Characterization of Collagen Solutions Conductivity and viscosity are the most important solution parameters that influence the electrospraying process.\(^{19,21}\) Figure 1 shows the conductivity of collagen solutions with and without salts as a function of acetic acid concentration. By increasing the acetic acid concentration, the conductivity decreased regardless of the presence/absence of salts. Addition of NaCl or CaCl\(_2\) increased the conductivity dramatically compared to plain collagen, with NaCl more effective at increasing conductivity compared to CaCl\(_2\). Note that the conductivities of 0.25 w/v% NaCl and CaCl\(_2\) without collagen were 5800 and 4740 \(\mu\)S/cm, respectively, which were almost 2-fold greater than the values in the presence of collagen, indicating binding of ions to the collagen molecules. Conductivity increased with NaCl concentration in the range of 0.25 to 1 w/v%; however, this increase was not proportional especially when the acetic acid concentration was low. Figure 2 shows viscosity of the collagen solution as a function of acetic acid concentration with or without 0.25 w/v% NaCl or CaCl\(_2\). A sharp decrease in viscosity was observed upon increasing acetic acid concentration or adding salt, which can partially be explained by a change in the size of aggregates and zeta potential that alters electrostatic repulsion between collagen molecules. However, those contributions were not sufficient, as shown next, for understanding dramatic change in the solution viscosity. Thus, alteration in the secondary and higher dimensional structure was most likely to affect intermolecular attraction. Helix-coil transition is known to occur for collagen upon increasing salt concentration. For example, the concentration threshold of CaCl\(_2\) at 25°C was reported as ca. 1.6 w/v% for a 0.05 w/v% collagen solution.\(^{28}\) Although the salt concentrations in our experimental condition were much lower than this, structural perturbation was assumed to alter interactions between the collagen molecules.

Figure 3 shows zeta potential variations as a function of acetic acid concentration with or without salts. In the absence of salts, the collagen aggregates exhibited negative zeta potentials and the absolute values increased with acetic acid concentration, which indicates association of acetate ions to positively charged residues of collagen molecules. In the presence of salts, the zeta potentials became positive. Thus, both sodium and calcium ions were most likely to be bound to the collagen molecules. A decrease in the zeta potential values with increasing acetic acid concentration could be produced by an increased association of acetate ions. As a result, increase in acetic acid concentration produced an opposite effect to the absolute value of the zeta potential in the absence and presence of salts. In the absence of salts, enhanced repulsion between the aggregates with increasing acetic acid concentration was expected, while repulsion between the aggregates should be weakened upon increasing acetic acid concentration in the presence of the salts.

The collagen solutions were not totally transparent, although there were no precipitations, suggesting heterogeneous solution structure. DLS studies suggested that collagen molecules formed loose aggregates and the averaged size was in the range of 1.0 to 1.3 \(\mu\)m regardless of acetic acid concentration (Supplemental information, Fig. S1). Addition of NaCl or CaCl\(_2\) slightly decreased and increased the size of the aggregates, respectively; however, the differences were only...
marginal. Nevertheless, increase in the size in the presence of calcium ions might be reasonable, because ionic cross-linking must be formed via calcium ions. Also, note that there was uncertainty in the size of the aggregates, because increase in the microviscosity around the aggregates may cause overestimation of the aggregate size.

Figure 4 shows information on the secondary structure of collagen obtained from analysis of the CD spectra (Supplemental information, Fig. S2). In the absence of salts, acetic acid concentration did not have significant effect on the secondary structure. However, in the presence of NaCl or CaCl₂, an increase in helix content was confirmed, notably in the presence of CaCl₂, although the amount of salts added in these experiments was not enough to cause the macroscopic helix-coil transition. Thus, a decrease in viscosity in the presence of salts could mainly be explained by the structural alteration, which should affect intermolecular interactions.

**Characterization of Collagen Structure Using Casted Membrane**

The FT-IR spectra (Supplemental information, Fig. S3) of native collagen membranes displays bands at 1654, 1554, and 1240 cm⁻¹, which are characteristic of the amide I, II and III bands. As the concentration of acetic acid increased, amide I, II and III were shifted to 1640, 1540, and 1290 cm⁻¹, respectively. The presence of an unordered conformation referred to as random coils produced an absorption peak at 1648 cm⁻¹. In the presence of CaCl₂, FT-IR observation indicated that collagen molecules bind to CaCl₂ through carboxy oxygen atoms and amino nitrogen in the collagen molecule. NaCl was confirmed to participate in structural alterations of collagen molecules.

XRD patterns (Supplemental information, Fig. S4) of the native collagen membrane had two diffraction peaks, one sharp and one broad, at diffraction angles of ca. 7.5° and 20.1°, respectively. The former corresponded to a periodic structure of collagen molecules in the dried state, and the latter was due to a disordered structure. The peak at 7.5° was not observed for membranes prepared using acetic acid solution regardless of the presence of salts, indicating that the collagen structure was loosened by addition of acetic acid. The diffraction pattern for collagen membrane in the presence of CaCl₂ and NaCl revealed the presence of unbound salt crystals.

**Preparation of Collagen Nanoparticles Using ESD**

The effect of acetic acid and salt dependency on the size of collagen nanoparticles and their structural morphology is shown in Figs. 5 and 6. Electrospraying of a collagen solution is known to form spinning fibers, which also occurred in our study when acetic acid solutions were used (Supplemental information, Fig. S5). However, the structural transition of collagen molecules in the presence of salts made the production of monodispersed collagen nanoparticles by electrospraying more feasible. The predominant particles produced had a caved structure, which is typical for particles produced by drying droplets. The collagen nanoparticles obtained by addition of NaCl or CaCl₂ yielded quasi-monodispersed particles with average diameter of ca. 900 nm or 693 nm, respectively, with 50 v/v% acetic acid. As the concentration of acetic acid increased up to 70 v/v%, particle size decreased, which probably was related to the decrease in solution viscosity in addition to the structural perturbation of collagen molecules. Because NaCl solution viscosity and conductivity were lower and higher, respectively, than those of CaCl₂ solutions at identical acetic acid concentrations, particle size was expected to be smaller when NaCl solutions were used. However, particle size was always larger in the presence of NaCl compared to CaCl₂ at identical acetic acid concentrations. Thus, an alteration in the secondary structure in the presence of salts seems to have a great effect on particle size. At 90 v/v % acetic acid, the particles tended to aggregate easily regardless of salt type, probably due to an increase in hygroscopicity.

Although addition of salt did not affect aggregation state of collagen molecules in the solution significantly, it enabled formation of dried particles after ESD. The most important effect of the added salts was likely to be increase in the conductiv-
ity, because it helps scission of the solution into fine droplets. Perturbation in the secondary structure of the collagen molecules may be another reason of the particle formation.

FT-IR spectra of collagen nanoparticles obtained after spraying revealed that the amide III peak was altered significantly in the presence of NaCl, but not with CaCl₂. The spectra of collagen nanoparticles exhibited a trend similar to that of the membrane. The electrosprayed collagen nanoparticles in the presence of salts possessed an amorphous structure as confirmed by the XRD analysis. Although NaCl was found to crystallize during electrospraying, only trace amounts of salt crystals were found when CaCl₂ was used. This may be explained simply by difference in the glass-forming ability of the salts. There were no indications of the stronger interaction of calcium ions to collagen molecules compared to sodium ions. However, enhanced alteration in the secondary structure in the presence of calcium ions might lead to higher entrapment efficiency of the salt molecules. Our previous investigation

Fig. 5. SEM Images of Electrosprayed Collagen Particles Prepared from Various Concentrations of Acetic Acid Solutions in the Presence of 0.25w/v% NaCl

Acetic acid concentrations were (a) 50 v/v%, (b) 60 v/v%, (c) 70 v/v%, and (d) 80 v/v%. Mean diameter is indicated in the figure.

Fig. 6. SEM Images of Electrosprayed Collagen Particles Prepared from Various Concentrations of Acetic Acid Solutions in the Presence of 0.25w/v% CaCl₂

Acetic acid concentrations were (a) 50 v/v%, (b) 60 v/v%, (c) 70 v/v%, and (d) 80 v/v%. Mean diameter is presented in the figure.
using chitosan revealed effect of each solution and operational condition on the particle formation during the electrospraying, in which solution viscosity and conductivity were shown to be the most dominant factors. When protein/peptide molecules are used as materials, its secondary structure appears to be an additional important factor to influence the particle formation behavior.

**Encapsulation of THP in Collagen Particles Using Coaxial ESD** To investigate the ability of collagen particles as a drug carrier, THP was encapsulated in collagen particles. Figure 7a shows collagen/THP = 10/2 particles produced by electrospraying a collagen/THP aqueous solution containing 50 v/v% acetic acid and 0.25 w/v% CaCl₂, in which many THP crystals were found. However, use of a coaxial nozzle allowed the crystalline THP to become entrapped in the collagen particles as shown in Fig. 7b. The operational conditions were optimized to obtain the particles with a diameter of ca. 2–3 µm, which is suitable for pulmonary drug delivery. Collagen molecules in these particles were cross-linked using glutaraldehyde vapor to obtain slightly aggregated particles (Fig. 7c). The collagen/THP particles exposed to glutaraldehyde vapor showed two distinct peaks at 1653 cm⁻¹ (N–H) and 1534 cm⁻¹ (N–H) in the FT-IR analysis indicating ionic cross-linking occurred (Supplementary content, Fig. S6). XRPD measurements revealed that THP was in the crystalline state in all cases.

Results of a dissolution study of collagen/THP particles are presented in Fig. 8. Intact THP powder dissolved immediately in phosphate buffer solution at pH 7.0. The collagen/THP particles (produced by the coaxial nozzle, Fig. 7b) without the cross-linking released ca. 47% THP immediately after contact with the aqueous media; however, the remaining was not released during the experimental period. Thus, approximately half of the THP molecules were expected to be located deep within the collagen particles. Further suppression of the release behavior was observed for the cross-linked particles that released THP slowly up to 31% after 2h, indicating that release of the drug from the collagen particles can be controlled by cross-linking. This observation indicated that collagen is an inert and biodegradable drug carrier with controllable release properties, which may be used for pulmonary drug delivery.

**Conclusion** Collagen nanoparticles were prepared using an ESD method. Although particulate formulation was not obtained using an acetic acid solution of collagen, addition of salts altered the solution properties and the secondary structure of collagen to enable formation of nanoparticles. THP was entrapped in collagen nanoparticles using a coaxial spray nozzle to achieve controlled release from the carrier. The electrospray technique is a relatively simple single-step process that does not expose
proteins to high temperatures or organic solvents, and thus can be used to prepare biodegradable nanoparticles as carriers for drug delivery.

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References