



## Effect of aggregation behavior of gelatin in aqueous solution on the grafting density of gelatin modified with glycidol

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### ABSTRACT

The effect of aggregation behavior of gelatin in aqueous solution on the grafting density of glycidol grafted gelatin polymers (GGG polymers) was investigated. The grafting density was measured using the Van Slyke method by calculating the conversion rate of free  $-NH_2$  groups of gelatin. The conversion rate reached peak values at 6% and 14% of the gelatin aqueous solution. SEM micrographs displayed a series of structural transitions (i.e., spherical, spindle, butterfly, irregular and dendritic aggregates) at varying concentrations from 2% to 16% (w/w) at an interval of 2% (w/w). The spindle aggregates reappeared at the concentrations of 6% and 14%. Viscosity measurements indicated that the physicochemical properties of the gelatin solution had changed with increasing concentration. UV and CD analysis indicated that hydrophobic interactions competed with hydrogen bonding, and the random coils partly transformed to  $\beta$ -sheet structure by changing the concentration. Zeta potential and pH data confirmed the increasing electrostatic repulsion associated with increasing the hydrophobic region. XPS analysis revealed that the elemental composition of the gelatin particle surface changed with variation in the aggregate structure, determining the monotonic variation of the grafting density with increasing concentration. Results demonstrate that aggregation behavior of gelatin in aqueous solution plays a crucial role in deciding the grafting density of gelatin modified products.

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### 1. Introduction

Gelatin, a water-soluble protein produced by the hydrolysis of animal collagen, has been widely used as a biomaterial in pharmaceutical and medical applications because of a range of favorable properties such as biocompatibility, biodegradability and bio-safety [1–3]. Gelatin solutions with concentrations covering the dilute and semidilute solution zones have been studied to explain the self-aggregation of gelatin in aqueous solution [4–8]. It is known that the aggregation process of gelatin is caused by intermolecular interactions and intramolecular folding [9–11]. Self-aggregation of gelatin gives rise to large scale structures above a certain concentration. Such structures have an effective molar mass higher than those of single chain, which leads to an increase of incompatibility with glycidol [12]. Moreover, the chemical reactions abilities in the aggregated structures between free  $-NH_2$  groups of gelatin and glycidol containing epoxy groups are largely limited and the conversion rate of free  $-NH_2$  groups in gelatin is incomplete.

Chemical grafting or cross-linking of gelatin is an effective way to introduce stable covalent bonds between protein segments to obtain a modified product which provides greater control of chemical and physical properties compared to natural polymers [13–15]. Grafting density plays a crucial role in deciding the physical and chemical properties of gelatin systems. In literatures, the grafting ratio has been studied by changing the reaction conditions including temperature, pressure, solvent and materials ratio [16–18]. However, the effect of aggregation behavior of gelatin in aqueous solution on the grafting ratio of gelatin has not been thoroughly studied.

In the present work, chemical modification of gelatin by glycidol at varying the gelatin concentration in water from 2 to 18% (w/w) was studied. The grafting density (= a decrease in number of  $-NH_2$  groups per unit mass) of glycidol grafted gelatin polymers (GGG polymers) is investigated using the Van Slyke method by measuring the conversion rate of free  $-NH_2$  groups of gelatin [19]. The evolution of the aggregate morphology of gelatin with concentration variation is visualized by scanning electron microscopy (SEM) micrographs. The impact of aggregation behavior of gelatin in aqueous solution on the grafting density of GGG polymers is analyzed by ultraviolet (UV) absorption spectra, circular dichroism (CD) analysis, pH values, zeta potential and X-ray photoelectron spectroscopy (XPS) measurements.

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## 2. Experimental

### 2.1. Material

All materials including gelatin (type A obtained from pigskin, having an approximate  $M_w$  of 50,000 and isoelectric point at pH = 8 determined by fluorescence measurements) and glycidol, were obtained from China National Medicine Corporation without further purification or treatment.

### 2.2. Preparation conditions

Gelatin was dissolved in distilled water (2–18% w/w) separately, and after 3 h, the gelatin solution was heated to 40 °C to ensure complete dissolution. The pH of each solution was adjusted to 10.0 by NaOH (2.0 mol/L) solution. The glycidol was then slowly added to the gelatin solution at 40 °C with stirring at predetermined glycidol/gelatin ratios (1:1, mol:mol). During reaction, the pH of these solutions was monitored using a pH-meter and held constant by the dropwise addition of NaOH solution (2.0 mol/L). After reacting for 12 h, the solutions obtained were used for viscosity (proRheo R180, Germany), pH (ESC Co., China) and zeta potential measurement (model: ZC-2000, Microtec, Japan) at 40 °C. Then, the solutions were cooled to 5 °C for weight and the content of free  $-NH_2$  groups was tested at 40 °C by the Van Slyke method.

### 2.3. SEM observation

Taking care to contrast the difference of aggregate structure of the gelatin particles in different solution concentrations, the same quantity was used for the different solutions at 40 °C. The details of the measurement can be found in our previous papers [20,21]. All the dried samples were coated with gold (~20 nm thickness) using a Sputter Coater SCD-005 (TEC Co., England), and then observed under a Quanta-200 ESEM (FEI Co., Holland).

### 2.4. XPS analysis

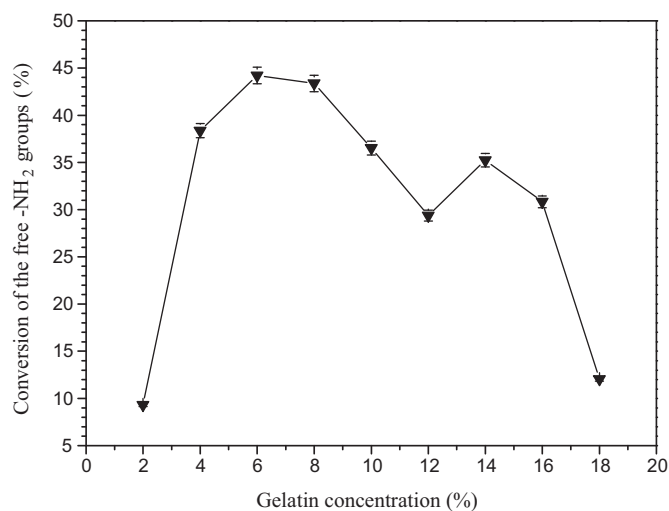
XPS spectra were taken using a Thermo Fisher Scientific ESCALAB 250 spectrometer (England) under the Al  $K\alpha$  line (1486.6 eV). The high-resolution scans were acquired at energy of 20 eV. Compositional analyses (0–1100 eV) and high-resolution scans of the C1s, N1s and O1s regions were carried out on all samples. Data treatment was performed with the Service Physics ESCAVB data reduction software.

### 2.5. UV analysis

UV spectra for the film samples formed from the gelatin solutions with different concentrations were recorded with a UV-2550 PC spectrophotometer (Japan) at room temperature. Corrections for the background were made. Broader features have been observed in the ultraviolet (the strongest at 278 nm).

### 2.6. CD analysis

Measurements were taken using a Jasco J-810 spectropolarimeter (Japan) equipped with a cavity (NESLAB RTE-111) and purged with  $N_2$  gas at a flow rate of 35 mL/min. The gelatin solution was placed in 0.1 cm path length cells for detection. Spectra were recorded from 190 to 250 nm with a resolution of 0.2 nm and an accumulation of six scans. The scan speed was 100 nm/min and the response time was 0.25 s.



**Fig. 1.** Conversion rate of free  $-NH_2$  groups with increasing gelatin concentration from 2% to 18% (w/w) at an interval of 2% (w/w). Error limit of conversion rate of free  $-NH_2$  groups is  $\pm 2.1\%$ .

## 3. Results and discussion

### 3.1. Chemical modification of gelatin by glycidol

The chemical modification of gelatin through chemical reactions between free  $-NH_2$  groups in gelatin and glycidol containing epoxy groups was performed in a water medium (Scheme 1). The concentration of the gelatin solutions ranged from 2% (w/w) to 18% (w/w) at an interval of 2% (w/w). The result of calculating conversion rate of free  $-NH_2$  groups suggests that the grafting density is significantly affected by the gelatin concentration. Fig. 1 shows that after the conversion rate of free  $-NH_2$  groups reaches a peak value at 6%, and then reaches another peak value at 14%. The results show that the variation of the conversion rate with increasing concentration is not monotonic, which cannot be explained by the standard viewpoint [22].

### 3.2. Aggregation morphology evolution

To visualize the evolution of the system morphology, SEM micrographs were taken at varying concentrations from 2% (w/w) to 16% (w/w) at an interval of 2% (w/w) of the gelatin aqueous solutions. The micrographs are shown in Fig. 2, and serve to illustrate how the microstructure develops with increasing concentration. Micrograph 2a shows some irregular spherical aggregates at the concentration of 2% (w/w) and a size distribution of gelatin particles ranging from 270 nm to 330 nm. However, after increasing the concentration to 4% (w/w), several spindle aggregates (about 1.5  $\mu m$ ) have appeared as indicated by the arrow shown in micrograph 2b. It can be seen in micrograph 2c that the spindle aggregates diameter has increased (about 2  $\mu m$ ), and the shape has more distinct at 6% (w/w) compared to micrograph 2b. A series of change in aggregation morphology of the gelatin particles was observed by SEM, including irregular aggregates (about 400–500 nm), network aggregates, butterfly aggregates and irregular aggregates in which the surface structure was more like a sepak takraw (about 1.0  $\mu m$ ), as shown in micrographs d–f. However, after increasing the concentration to 14% (w/w) the spindle aggregates (about 3–4  $\mu m$ ) reappeared (Fig. 2g). Although the shape of the spindle aggregates at 14% is in quite similar to the morphology at 6%, the size of the aggregates increased, which is an unfavorable factor for chemical reaction because of the larger scale structure. Finally, micrograph 2h displays the microstructure at 16% (w/w) (dendritic aggregates, about

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