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The comparison of physicochemical properties of four Cross-linked sodium hyaluronate gels with different cross-linking agents

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Keywords: cross-linked sodium hyaluronate gels, different cross-linking agents, dynamic viscosity, intrinsic viscosity, enzyme-resistant degradation properties *in vitro*

Abstract: Purpose The physicochemical properties of four cross-linked sodium hyaluronate gels (CHA) with different cross-linking agents were compared in order to research out the different stability and Enzyme-resistant degradation properties of these CHA gels. **Methods** The CHA hydrogels were prepared with different cross-linking agents, such as PEG20000, PDE, BDDE and ADH. The optimal reaction conditions were determined by single factor experiment. Dynamic viscosity was tested by Stabinger method. Intrinsic viscosity was determined by Uzziah's viscosity method. The enzyme-resistant degradation properties *in vitro* of CHA-gels were analysed by carbazole and spectrophotometry. **Results** The concentrations of NaOH/HCl, concentrations of HA and the ratio of cross-linking agent to HA are major factors of conditions which influenced the physicochemical properties of CHA gels. PDE-CHA and PEG20000-CHA gels possess better Dynamic viscosity, PDE-CHA gel has also better intrinsic viscosity, ADH-CHA and BDDE-CHA gels get better Enzyme-resistant degradation properties than PEG20000-CHA and PDE-CHA gels. **Conclusion** The CHA-gels prepared under optimal reaction conditions have different physical and chemical properties, which set foundation for developing double cross-linked or multifunctional gels with both excellent stability and enzyme-resistant degradation properties.

Introduction

Hyalouronic acid(HA), a high molecular weight, acidic polysaccharide typically composed of the alternating disaccharide GlcUA(β1→3)GlcNAc[1]. It has wonderful biocompatibility, but natural hyaluronic acid is easy to degrade by hyaluronidase(HAase) in vivo[2,3].

At present, at home and abroad, researches carried out by cross-linking HA gel with different cross-linking agents in order to get better viscoelasticity and Enzyme-resistant degradation properties, and maitain the original biocompatibility [4-7]. The marketted products of CHA gels are Allergan's Hylaform® gel of U.S.A[8], CORNEAL's SKGEL® gel of France[9], Q-Med AB's Restylane® gel of Sweden[10] and so on. The cross-linking agents of these CHA gel products such as biscarbodimide (EDC) , divinyl sulfone(DVS) [11-13], are all allergenic and toxic more or little. PEG20000, PDE, ADH and BDDE are cross-linking agents with the characteristics of lower-toxicity and will have broad application in pharmaceutics and cosmeceuticals [14-16]. Different physicochemical properties of four CHA gels preparing with PEG20000, PDE, ADH and BDDE were compared in this paper.

MATERIALS AND METHODS

MATERIALS

PEG20000(SIGMA); Adipic acid dihydrazide, ADH(SIGMA); Poly diglycidyl ether, PDE(SIGMA); 1,4-Butanediol diglycidyl ether, BDDE(Alfa Aesar); Hyaluronidase (SIGMA); Medical level sodium hyaluronate dry powder(09120810)(Shandong FuRuiDa biological chemical Co., LTD); Carbazole(Chinese medicine group Shanghai chemical reagent company); The standard glucuronic acid(Shanghai biological technology Co., LTD); Folin-Ciocalteu's phenol(Shanghai Ruji biological technology development Co., LTD); NaOH; HCl; Na_2HPO4; NaH_2PO4; Anhydrous alcohol; phenol;

FA1204B electronic balance (Shanghai precision &scientific instrument Co., LTD); 85-type 1 constant temperature and heating magnetic blender (changzhou guohua instrument plant); 723 N uv-vis spectrophotometer; HH-4 display constant temperature water-bath pot; TGL-16 C high-speed centrifuge; LDZX-50 a KBS vertical pressure steam sterilization pot; Mooney viscometer having rotor syringe; Ubbelohde viscometer.

METHODS

Preparation of Cross-linked hyaluronate gels

The cross-linked hyaluronate gels were prepared by the following process: A certain weight of medical level sodium hyaluronate dry powder was dissolved in the solution of 0.001mol/mL~ 0.1 mol/mL NaOH or HCl, then different cross-linking agents which are PEG20000, PDE, BDDE and ADH were added into the solution. Churned the mixtures for hours to make the CHA-gels homogeneous. The pH of CHA-gels was adjusted at 7.0~7.3. At the end, CHA-gels were heat-treated at 115°C ~121°C for 8 min~30min.

Comparisons of stability of CHA gels under different conditions

Taking dynamic viscosity and intrinsic viscosity[18] as the evaluation standards of stability of CHA gels, the factors which influenced the stability of CHA were chosen, such as the different cross-linking agents, the time of reaction, the reaction temperatures, the concentrations of HA, the concentrations of NaOH and HCl, the ratios of cross-linking agents to HA (g/g).

Comparison of stability of CHA gels with different time of reaction

The reacting time of preparing CHA-gel was set from 2h~36h. The selected cross-linking agents were PEG20000, PDE, BDDE and ADH. Others conditions were set with definite parameters.

Comparison of stability of CHA gels by different temperatures

The choosing temperature scope of preparing CHA-gel were from 0˚C~100˚C. The selected cross-linking agents were PEG20000, PDE, BDDE and ADH. Others conditions of preparation were set with definite parameters.

Comparison of stability of CHA gels by different concentrations of NaOH and HCl

The concentrations of NaOH/ HCl agents were set from 0.001mol/mL~ 0.1 mol/mL. The selected cross-linking agents were PEG20000, PDE, BDDE and ADH. Others conditions were set with definite parameters.

Comparison of stability of CHA gels under different concentrations of HA

The choosing concentrations of HA was from 0.5%~2.5%.The selected cross-linking agents were PEG20000, PDE, BDDE and ADH. Others conditions were set with definite parameters.

Comparison of stability of CHA gels with the different ratios of cross-linking agent to HA

The ratio of cross-linking agent to HA was set from1:20~1:2.5(g/g). The selected cross-linking agents were PEG20000, PDE, BDDE and ADH. Others conditions were set with definite parameters.

Comparison of stability of CHA gels by different conditions of sterilization

The prepared PEG20000-CHA, PDE-CHA ,BDDE-CHA and ADH-CHA gels were categorized into two groups, one was treated at 121°C for 8 min. The other one was treated at 115°C for 30min.The parameters of dynamic viscosity and intrinsic viscosity were compared respectively.

The determination of viscoelasticity of CHA-gels.

The dynamic viscosity of CHA-gels prepared by different cross-linking agents were tested by Stabinger method. The intrinsic viscosity of CHA-gels prepared by different cross-linking agents were determined by Uzziah's viscosity method[18].

Examination of enzyme-resistant degradation properties of CHA-gels *in vitro*

Carbazole method[18]. 4g different CHA-gels which were prepared as above were analysed respectively. The gels were added with 2 ml of 856 U/ml HAase，at 37 ˚C, reacting for 65hr. Let the volume to 5 ml with PBS buffer solution(pH 7.1),then pipetted 1ml of the gel-extract mixtures adding with 4ml of absolute ethyl alcohol respectively to centrifugate for 15 min,at the speed of 10000 r/ min. Pipetted 2 ml supernatant liquid which let the volume to 5mL by PBS as solution I.

On the other hand, 15g different CHA-gels which were prepared as above were hydrolyzed by 10ml sulfuric acid solution (0.5 mol/L) for 20 min in the boiling water bath. Metered the volume to 100 ml with PBS buffer solution (pH 7.1) as solution II. 1mL of second solution was used to be analyzed.

The Carbazole and sulphuric acid spectrophotometry was used to analysed the content of GlcA,the numerical value of resistance to enzymatic degradation in vitro(R) was computed by the following formula:

$$R = 1 - 15/64 \times A/B$$

A—content of GlcA in the I solution. (mg/ml);

B—content of GlcA in the II solution. (mg/ml).

RESULTS and DISCUSSION

Stability of CHA gels under different times of reaction

The researched results of reaction time of CHA-gel indicate that the stability of the CHA-gel is increasing with the time of reaction. The reaction probably ends by 4hr because of the possession of the approximate data of viscoelasticity of the gel by the end of 4hr and 6hr. Results are shown in Figure 1.

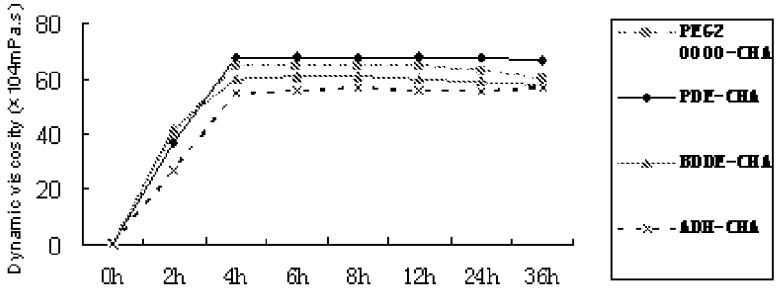

Figure 1 Dynamic viscosity (×10⁴mPa.s) of CHA gels at different times

Stability of CHA gels under different reaction temperatures

The stability of different CHA-gels sharply go up between 0˚C and 20˚C,they remain steady between 20˚C~40˚C,and then drop at 60˚C~100˚C. PEG20000-CHA and PDE-CHA gels have better heat-stablility than BDDE-CHA and ADH-CHA All the process of CHA preparation should be below 40˚C in order to get gels of excellent stability or physicochemical properties. Comparative results are shown in Figure 2.

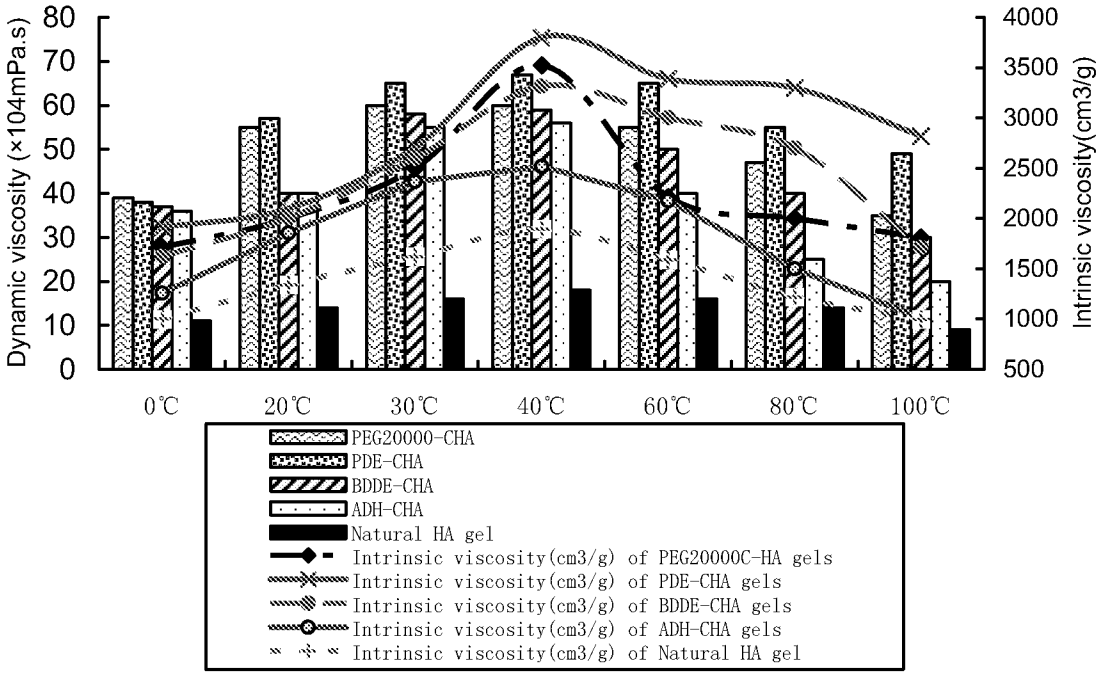

Figure 2 Dynamic viscosity (×10^4mPa.s) and Intrinsic viscosity(cm^3/g) of CHA gels at different temperatures

Influence of different concentrations of NaOH and HCl on stability of CHA gels

The stability of the CHA-gels are decreasing along with the increasing concentration of NaOH or HCl, for the simple reason that CHA-gels are easily degradable in the high pH as well as natural hyaluronic acid.

The dynamic viscosity of PEG20000-CHA prepared with NaOH solution is higher than with HCl solution,The dynamic viscosity of PDE-CHA, BDDE-CHA and ADH-CHA gels prepared with HCl solution are higher than with NaOH solution. The results for the dertermination of intrinsic viscosity show that: PDE-CHA> PEG20000-CHA and BDDE-CHA> ADH-CHA.

The optimal conditions of preparing PEG20000-CHA gel is 0.001mol/mL NaOH, The optimal conditions of PDE-CHA, BDDE-CHA and ADH-CHA gel preparing is 0.001mol/mL HCl. Comparison results are shown in Figure 3 and Figure 4.

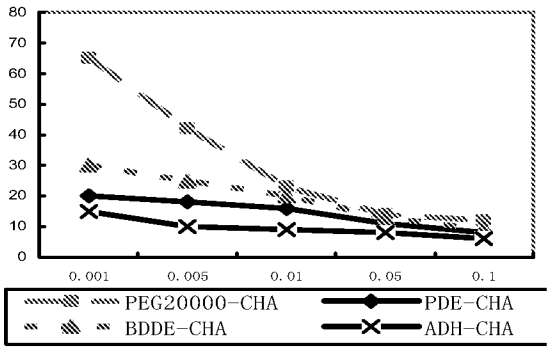

Figure 3 Dynamic viscosity (×10^4mPa.s) of CHA under different concentrations of NaOH(mol/mL)

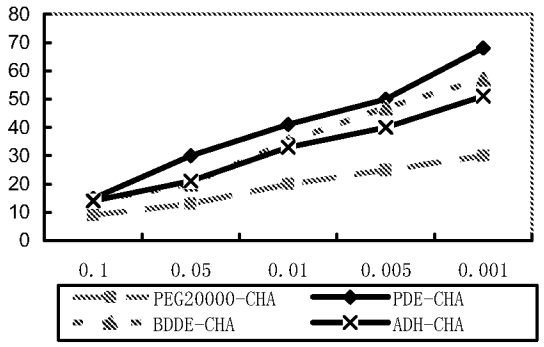

Figure 4 Dynamic viscosity (×10^4mPa.s) of CHA gels under different concentrations of HCl(mol/mL)

Influence of the different ratios of cross-linking agent to HA on stability of CHA gels

The dynamic viscosity of the CHA-gel steadily increased between 1:20 and 1:10,but the figures fallen at the ratio of 1:2.5. The examination results of intrinsic viscosity are PDE-CHA> PEG20000-CHA and BDDE-CHA> ADH-CHA.

Comparison of stability of CHA gels with different concentrations of HA

The dynamic viscosity and intrinsic viscosity of CHA-gels are increasing along with the growing concentration of HA, dynamic viscosity rising from about 10^5mPa.s to over 10^6mPa.s, intrinsic viscosity rising from 2517 cm^3/g to 5241cm^3/g.

Stability of CHA gels under different conditions of sterilization

The dynamic viscosity of CHA gels after sterilization are just about 1/3-1/2 persentage of non-sterilizated CHA gels, but much high than natural HA gel.The keeping proportions of intrinsic viscosity are at most 80% percentage after sterilization. The steady dynamic viscosity numbers of gels indicate that PEG20000-CHA, PDE-CHA and BDDE-CHA gels have better heat- stability than ADH-CHA and natural HA gels. After 115˚C sterilization for 30min, the dynamic viscosity of ADH-CHA gels, along with the intrinsic viscosity of BDDE-CHA and ADH-CHA gels went down to the bottom. Comparison results are shown in Figure 5, Figure 6.

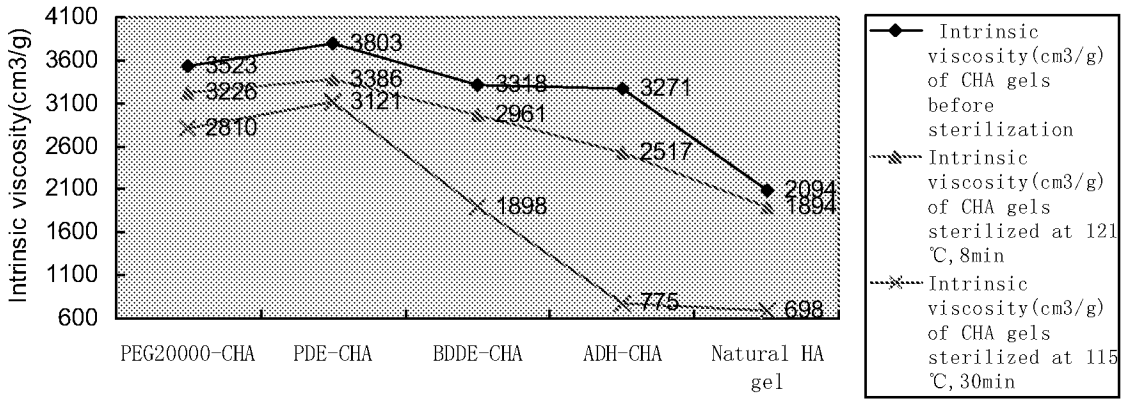

Figure 5 The Intrinsic viscosity(cm^3/g) of CHA gels before and after sterilization

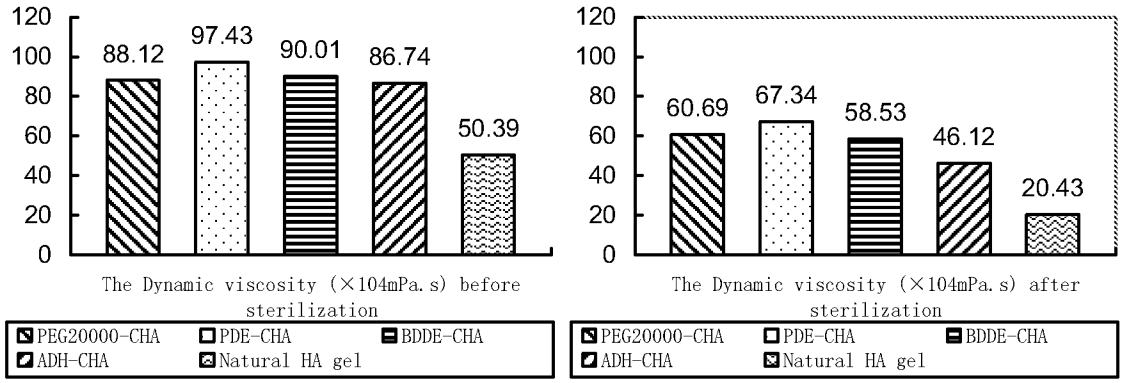

Figure 6 Dynamic viscosity (×10^4mPa.s) of CHA gels before and after sterilization

The dynamic viscosity and intrinsic viscosity decreased after sterilization indicate that the preparing process of CHA still should be optimized, in order to have better stability of cross-linked sodium hyaluronate gels.

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