Immobilization of Concanavalin A to Glucose-Containing Polymers

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SUMMARY: Sol-gel reversible hydrogels sensitive to environmental glucose concentration were prepared using concanavalin A (Con A) and glucose-containing polymers. Since the components of the hydrogels in the sol state can be released to the environment through pores of the dialysis membrane, it was necessary to immobilize Con A to the glucose-containing polymers. Con A was immobilized by two different approaches. First, vinyl groups were introduced to Con A so that it can participate in the vinyl polymerization of allyl glucoside. Second, glucose-containing polymers containing chemically reactive pendant groups were synthesized so that Con A could be immobilized to the preformed polymers. Both approaches resulted in effective immobilization of Con A to the glucose-containing polymers, but the second method appeared to be better in terms of maintaining the bioactivity of Con A.

Introduction

The sol-gel phase transition behavior of hydrogels composed of Con A and glucose-containing polymers have been studied in our previous studies[1,2]. The sol-gel phase-reversible hydrogel systems have the potential for self-regulated insulin delivery[3]. The reversible sol-gel phase transition in response to changes in the environmental glucose concentration resulted in modulated insulin delivery. In most of the hydrogel systems used for modulated insulin delivery, hydrogel components were kept inside dialysis membranes (MW cut-off 50 000) to prevent them from leaking in the sol state. The insulin release experiments, however, showed a gradual increase in the insulin release rate, and this was most likely due to the leakage of the hydrogel components. Concanavalin A (Con A) and glucose-containing polymers may have leaked out in the sol state. Leakage of Con A should be prevented, since it is known to be immunotoxic[4,5]. The leakage of Con A can be eliminated or minimized by immobilizing it to glucose-containing polymers through covalent bonding. The supramolecular structure of the Con A-glucose-containing polymers, as described in Fig. 1, is expected to prevent any leakage through the pores in dialysis membranes.

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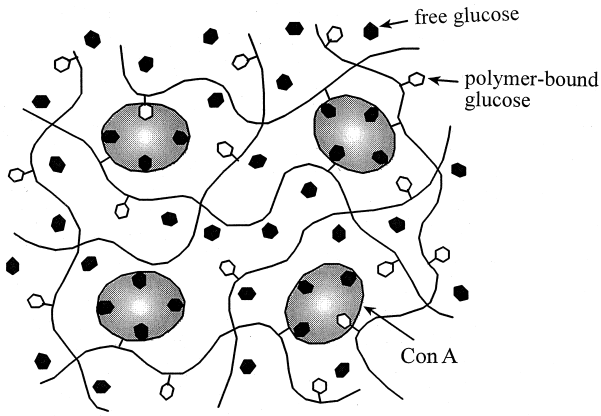

Fig. 1: Representation of a glucose-sensitive hydrogel that consists of Con A immobilized to glucose-containing polymer chains. Con A molecules are covalently attached to the glucose-containing polymers.

Immobilization of Con A to glucose-containing polymers can be achieved by at least two different approaches. First, Con A can be modified to possess vinyl groups which can participate in the vinyl polymerization of allyl glucoside, used for making glucose-containing polymers. Incorporating biomolecules into polymers during polymer synthesis requires protection of the biomolecules from the polymerization environment[6,7]. Second, Con A can be grafted to the preformed glucose polymer with reactive groups[8-12]. A number of researchers tried to incorporate Con A to polymers and devices to prevent it from direct contact with body fluids[13-15]. Con A needs to be covalently bound to the support material while maintaining its native glucose-binding activity. For this reason, we examined the above mentioned two approaches for Con A immobilization to glucose-containing polymers.

Materials and Methods

Materials: Acrylamide (AM), 2,2'-azobisisobutyronitrile (AIBN), 2,2-dimethoxy-2-phenyl-acetophenone, acryloyl chloride, *N*-hydroxysuccinimide (NHS), 2,6-di-*tert*-butyl-4-methylphenol (BMP), and *N*-vinylpyrrolidone (VP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). VP was purified by distillation under reduced pressure before use. Triethylamine was obtained from Fisher Scientific Co. (Pittsburgh, PA). Glycidyl acrylate (GA) was purchased from Polysciences, Inc. (Warrington, PA). PEG acrylate 2-(succinimidyloxycarbonyl)ethyl ether (APEG-NHS, MW_{PEG} 3 400) and PEG methyl ether 4-nitrophenyl carbonate (MPEG-NPC, MW 5 000) were obtained from Shearwater Polymers, Inc. (Huntsville, AL). Allyl glucoside (AG) was synthesized as described before[1,2]. Con A

(Type IV, essentially salt-free, lyophilized powder) was purchased from Sigma Chemical Co. (St. Louis, MO) and used as received without further purification. Spectra/Por® dialysis membranes were purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA).

Polymerization with functionalized Con A: APEG-NHS was used to functionalize Con A. The reactive and accessible amine groups on Con A surface react with APEG-NHS at pH 8.5. One end of APEG-NHS is designed to couple to Con A, while the other has a reactive group to participate in polymerization. The reactive PEG has an active ester (to react with lysine and other primary amino groups of Con A) and an acryloyl group (to incorporate the resulting PEGylated Con A into glucose-containing polymers). Schematic diagram for Con A immobilization during polymer synthesis is illustrated in Fig. 2. In the first step, Con A was functionalized with APEG-NHS. Con A solution (8 ml) was prepared in 0.1 M borate buffer (pH 8.5) by dissolving 100 mg of Con A. A calculated amount of APEG-NHS was slowly added to Con A solutions at room temperature. Molecular weights of Con A and APEG-NHS were 25 000 and 3400, respectively. Molar ratios of APEG-NHS to Con A were 1:1, 2:1, and 3:1. After 4 h of reaction, the mixtures were dialyzed against deionized distilled water at 4 °C for 4 days using Spectra/Por® dialysis membranes (MW cut-off 50 000; Spectrum Medical Industries, Inc., Los Angeles, CA). Some precipitates were observed during dialysis. The dialysate was lyophilized to obtain a white fluffy product. The functionalized Con A did not dissolve well in water, and aggregated particles remained in the solution. To overcome this problem, direct polymerization was attempted just after functionalizing Con A. After 1 h of reaction, 75 mg of VP and 24 mg of AG were added to the reaction mixture of Con A and APEG-NHS (Fig. 2).

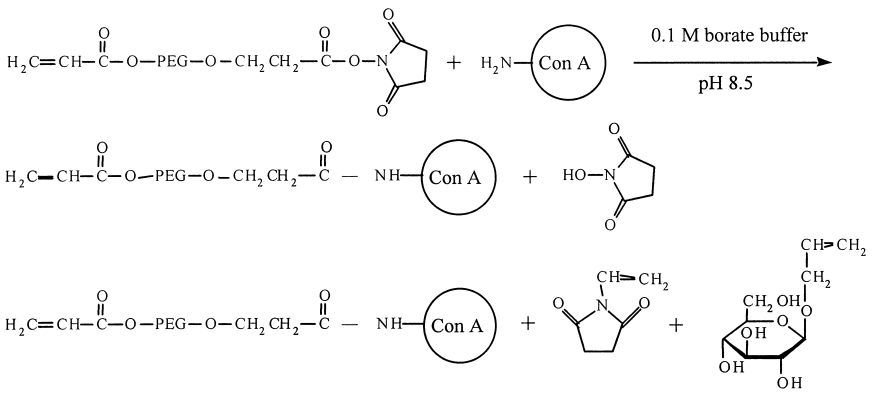

Fig. 2: Schematic representation of Con A modification with APEG-NHS (I) and Con A immobilization to glucose-containing polymer (II).

2,2-Dimethoxy-2-phenyl-acetophenone was used as a photoinitiator and the mixture was UV-irradiated at 366 nm for 20 h. The reaction mixture was dialyzed and then lyophilized. AG was also used in addition to APEG-NHS. The same molar ratios to Con A and the same reaction scheme were used. PEGylated Con A was also used instead of Con A.

Synthesis of *N*-(acryloyloxy)succinimide: *N*-(acryloyloxy)succinimide (NAS) was synthesized by following the method reported by Pollak and coworkers[8]. *N*-hydroxysuccinimide (11.5 g, 1.0 mol) and triethylamine (11 g) were dissolved in 150 ml of chloroform at 0 °C. Acryloyl chloride (10 g, 1.1 mol) was added dropwise over a 20 min period to the reaction mixture while stirring. After additional 20 min at 0 °C, the solution was washed with 80 ml of ice-cold water. Chloroform solution was separated and dried with anhydrous $MgSO_4$, and filtered. Five mg of BMP was added to the solution as a polymerization inhibitor, and the solution was concentrated to a volume of 30 ml in vacuo using a rotary evaporator and filtered. Ethyl acetate (3 ml) and *n*-hexane (20 ml) were added slowly with stirring to the chloroform solution, and the mixture was left to stand at 0 °C for overnight. The precipitated, colorless crystals were filtered off and washed with 40 ml of a mixture of cold *n*-hexane and ethyl acetate (4:1), 90 ml of the cold mixture (9:1), and finally twice with 40 ml of *n*-hexane. The crystals were dried in vacuo at ambient temperature to constant weight. The melting point of the obtained product (11.5 g) was 68.5-71.0 °C. The melting point of the product recrystallized from a mixture of *n*-hexane and ethyl acetate showed a good agreement with the literature value 69.5-71.0 °C. R_f values of TLC in the mixture of ethyl acetate and *n*-hexane (4:1) were 0.32 for *N*-hydroxysuccinimide, 0.84 for acryloyl chloride, and 0.74 for the product. The product showed the expected ^{1}H NMR spectral characteristics ($CDCl_3$): δ 2.85 (s, 4 H), δ 6.1-6.4 (m, 2 H), δ 6.6-6.8 (d, 1 H).

Preparation of reactive polymers: Copolymer of NAS and VP was synthesized by radical polymerization. NAS (0.096 g) and VP (3 ml) were dissolved in 37 ml of dimethylformamide (DMF). The concentration of NAS was 0.0142 M and the molar ratio of NAS to VP was 0.021. Another solution with [NAS]/[VP] = 0.053 was also prepared. The monomer solutions were purged with dry N_2 gas to remove air bubbles for 20 min. AIBN was added to the solutions as an initiator at the concentration of 0.5% (w/w) of monomers. Further N_2 gas purging was performed for 5 min. The reaction mixtures were sealed and heated in sealed vials for 20 h at 60 °C. Then they were concentrated in vacuo using a rotary evaporator and copolymers were precipitated in diethyl ether followed by filtration. The filtered copolymer was washed with diethyl ether and dried under vacuum for 24 h at room temperature. Assay

for the active ester content was carried out and verified the presence of NAS group in the polymers.

Reactive polymer containing AG group was prepared by following the same procedure described above. AG was added to the solution at the final concentration of 0.11 M, which gave the molar ratio of AG to VP as much of 0.16. The number-average molecular weights (M_n) of the resulting polymers were less than 3000. Another terpolymer was prepared using AM (Fig. 3). AM was used to increase the molecular weights of terpolymers instead of VP. Mole fractions of NAS in feed solutions were 0.02 and 0.05. The molar ratio of AG to AM was fixed to 0.13.

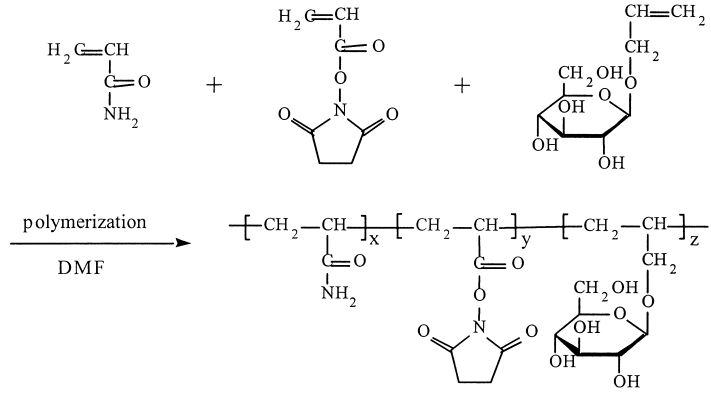

Fig. 3: Schematic representation of synthesis of a glucose-containing polymer with chemically reactive functional groups from acrylamide (AM), *N*-(acryloyloxy)succinimide (NAS), and allyl glucoside (AG).

Assay for the active ester content of reactive polymers: The active ester contents of the synthesized copolymers were assayed by reacting the copolymers with aqueous isopropylamine solution and measuring the absorbance of the anion of NHS. An absorptivity of 8600 l $mol^{-1}cm^{-1}$ was reported[16] for the NHS anion at pH 7.5. The reactive copolymers (70 mg, dried under vacuum at RT for 24 h) were dissolved in 5 ml of deionized distilled water in a test tube. A 50 µl aliquot of this solution was added into a test tube containing 3 ml of borate buffer (0.1 M, pH 8.0) and 50 µl of 1 M isopropylamine. After the reaction was completed (ca. 90 min), the absorbance of NHS anion was measured at 259 nm. A calibration curve was constructed by measuring the absorbance of NHS standard solutions containing isopropylamine. The active ester content of the reactive copolymer was calculated using the

equation of the calibration curve, $A = 0.0091c + 0.0116$, where A is absorbance at 259 nm and c is concentration of the NHS anion in μmol l^{-1}.

Results and Discussion

Polymerization with functionalized Con A: Immobilization of Con A to polymers requires bifunctional reagents. APEG-NHS has the acryloyl and succinimidyl ester end groups. Coupling of APEG-NHS to Con A results in Con A with a polymerizable vinyl group (Fig. 2). The extent of conjugation of APEG-NHS was measured by fluorescamine method. Unfortunately, however, the number of attached APEG-NHS could not be determined due to insolubility of functionalized product in aqueous solution. The same results were observed in repeated experiments with various concentrations of reactants. To solve this problem, functionalized Con A was used for polymerization immediately after preparation. After addition of comonomers and photoinitiator, the solution was irradiated with UV light. The polymerized products were used to make hydrogels in 0.1 M Tris buffer solution (pH 7.4) with 0.1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Those products also had some insoluble aggregates in the solution. This phenomenon might have been due to chemical crosslinking of the functionalized Con A. Photopolymerization might have also caused detrimental effect on Con A. It was reported that enzymes were deactivated during procedures requiring radical polymerization in enzyme-containing solutions of vinylic monomers[16,17]. Deactivation of enzymes was mainly caused by autoxidation of cysteine thiol groups. Although Con A has no cysteine residues, polymerization procedure may have led to unwanted denaturation of Con A. Heat evolved during photopolymerization may be one of the reasons for denaturation of Con A. The extremely low ratio of APEG-NHS to Con A in solution was not investigated, because low efficiency of immobilization could hinder the separation of product from unreacted Con A.

Characterization of reactive polymers: Immobilization of Con A to hydrophilic polymer was achieved by an alternative method utilizing reactive polymers containing active esters (Fig. 3). NAS, which was used as a bifunctional monomer, was synthesized from acryloyl chloride and NHS. The AG contents of the polymers were determined by using phenol-sulfuric acid method. The active ester contents of reactive copolymers were assayed by measuring the absorbance resulting from the anion of NHS. An absorptivity for the NHS anion was 9100 l $mol^{-1}cm^{-1}$ at pH 7.5 in aqueous isopropylamine solution. The amount of NHS anion (μmol of NHS per gram of dry polymer) released from the polymer was determined. The concentration of neutral NHS at pH 7.5 was ignored ($pK_a = 6.0$ for NHS). If

the molecular weight of the polymer is known, the number of active ester group per polymer chain can be calculated. For the calculation of mole fraction of AG in copolymer, the active ester groups were ignored and copolymers were assumed to be made of VP or AM and AG.

The compositions of reactive polymers are summarized in Table 1. At the beginning of experiments, VP was chosen as a comonomer to NAS, because poly(vinylpyrrolidone) is known to be biocompatible. Recently, Erout and coworkers studied kinetics and microstructure of radical-initiated copolymers of VP and NAS[18]. They reported that the average values for the reactivity ratios were $r_{NAS} = 0.27 \pm 0.04$ and $r_{VP} = 0 \pm 0.08$. Strong cross-propagation during the copolymerization of VP and NAS was suggested. In their study, however, the prepared copolymers of VP and NAS showed low molecular weights. The low molecular weight of reactive polymers was thought to be inappropriate to immobilize Con A, because of the low number of active groups per polymer chain. One active ester group per chain would be ideal for single-point attachment of Con A to polymer. Considering low crosslinking and coupling efficiency of the reaction, one may tolerate even two or more active groups per polymer chain. Incorporation of NAS to copolymers was related to the concentration of NAS in feed solutions. The numbers following NAS in Table 1 represent mole % of NAS in feed solutions. The molecular weight of reactive polymers greatly increased by introducing AM instead of VP into monomer mixture as in the case of poly(AM-NAS2-AG). The increased molecular weight of polymer resulted in increased number of active groups per polymer chain.

Table 1. Characteristics of the reactive polymers

Polymer	M_n	M_w/M_a	x_{AG}[a]	AE[b]	N_{RG}[c]
Poly(VP-NAS2)	4350	3.84	-	131	0.57
Poly(VP-NAS5)	4160	4.32	-	266	1.11
Poly(VP-NAS2-AG)	2520	3.36	0.083	164	0.41
Poly(VP-NAS5-AG)	2070	3.46	0.086	348	0.72
Poly(AM-NAS2-AG)	18000	2.04	0.041	160	2.88

[a] Mole fraction of AG; [b] active ester content (μequiv/g); [c] average number of reactive groups per polymer chain

It is hard to immobilize each Con A molecule to one single polymer chain (single-point connection) due to characteristics of the chemical reaction involved. It is possible that some Con A molecules are covalently linked to the glucose-containing polymers via more than one

point while others are not linked at all. Even though the single-point connection is achieved, the resulting hydrogel system is likely to have a complicated structure. Con A may form a tetramer at neutral pH and the glucose units of the polymer may bind to the binding sites of Con A. It may also be hard to separate the immobilized Con A from free Con A because of the polydispersity and properties of binding of the polymer to Con A. More advanced separation methods will be required rather than usual size-exclusive separation methods, such as dialysis and gel permeation chromatography. Use of low pH condition would be better than using neutral pH condition in dialysis or gel permeation chromatography due to low binding activity of Con A to carbohydrates. The next step is to test the glucose binding property of the Con A that is immobilized to the glucose-containing polymers.

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