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Controlled Curing of Adhesive Complex Coacervates with Reversible Periodate Carbohydrate Complexes

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Abstract

Periodate oxidation of carbohydrates with vicinal hydroxyl groups and aromatic *ortho*-dihydroxyphenyl groups has been employed extensively to initiate crosslinking or conjugation reactions in adhesive biomaterials. Periodate forms stable tridentate complexes with carbohydrates containing three appropriately configured hydroxyls, such as 1,2-O-Isopropylidene- α -D-glucofuranose, that are not appreciably oxidized relative to carbohydrates with vicinal hydroxyls and *ortho*-dihydroxyphenyl groups. In the presence of 1,2-O-Isopropylidene- α -D-glucofuranose the rate of periodate oxidation of dihydroxy containing compounds is controlled by the rates of association and dissociation of the periodate-carbohydrate complex. By varying the ratio of 1,2-O-Isopropylidene- α -D-glucofuranose to periodate the curing rate of adhesive complex coacervates was varied over a wide range.

Keywords

bioadhesive; complex coacervates; *ortho*-dihydroxyphenyl; dopa; periodate

INTRODUCTION

Periodate oxidation of vicinal hydroxyls in carbohydrates and *ortho*-dihydroxyphenyls (*o*-DHP) are well-studied reactions that have found broad utility in the life sciences. The oxidation products are reactive carbonyl intermediates used in subsequent conjugation or crosslinking reactions. In the adhesive biomaterials arena, several examples of adhesives have been reported based on the introduction of reactive aldehyde groups into polysaccharides, such as dextran or chondroitin sulfate, by periodate oxidation [1–4]. The activated polysaccharides are then reacted with polyamines, such as branched polyethylene glycol amines or chitosan, to form hydrogels. In other cases they are reacted directly with tissues [5].

The reactivity of *ortho*-quinones generated by periodate oxidation of *o*-DHPs has been exploited in several instances of naturally inspired adhesives that mimic aspects of the bioadhesives produced by mussels and Sandcastle worms. Proteins containing 3, 4-L-dihydroxyphenylalanine (dopa) isolated directly from the adhesive glands of mussels [6], synthetic dopa-containing polypeptides [7], and *o*-DHP containing polystyrenes [8] have

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been cross-linked by periodate oxidation. Similarly, addition of NaIO₄ to dopa-modified poly(ethylene glycol) resulted in gelling in less than a minute [9]. In the case of the Sandcastle worm, its densely and oppositely charged dopa-containing adhesive proteins served as a model for synthetic adhesive complex coacervates that are rapidly cured by periodate oxidation of *o*-DHP side chains [10–12].

When used to cure adhesives there are scenarios in which it is desirable to control the rate of *o*-DHP mediated copolymer cross-linking. A controlled curing reaction would allow more accurate positioning of the adherends before the adhesive sets, for example, and could also simplify packaging and delivery of a two-part adhesive. As one approach, Burke and coworkers [13] reported temperature controlled periodate cross-linking of *o*-DHP copolymers based on sequestering NaIO₄ into liposomes at ambient temperature. The liposomes were designed to melt near 37°C to release of NaIO₄ for rapid gelation of *o*-DHP copolymers at body temperature.

Mechanistically, periodate oxidation of both aliphatic and aromatic vicinal diols proceeds through intermediate bidentate iodoester complexes [14–16]. Formation of the reversible bidentate complexes is rapid; the rate-limiting step in the overall reaction is breakdown of the complex into the oxidation products. The stability of the intermediate coordination complex is dramatically increased with carbohydrates that have a third hydroxyl group appropriately configured to form a tridentate complex [17], the effect of which is to slow oxidative scission by several orders of magnitude. One such group of carbohydrates are the furanose derivatives. A well-studied example is 1,2-O-Isopropylidene- α -D-glucofuranose (IPGF) that forms a stable 3,4,6-tridentate complex with periodate at slightly basic pH (Fig. 1). IPGF is oxidized by periodate several orders of magnitude more slowly than ethylene glycol and other linear polyols [18]. We reasoned that a straightforward method to control the rate of periodate oxidation of *o*-DHP and subsequent cross-linking of adhesive complex coacervates would be to sequester the periodate in a reversible IPGF complex as illustrated in scheme 1. Competitive formation of a periodate IPGF complex would slow the rapid oxidation of *o*-DHP thereby allowing time for thorough mixing and application before full cure of the adhesive complex coacervate. Here, we demonstrate the rate of *o*-DHP mediated curing of adhesive complex coacervates can be varied over a wide range by varying the ratio of IPGF to periodate.

MATERIALS AND METHODS

Materials

Low-endotoxin, non-gelling gelatin (Mw 3–5 kDa) was a gift from Gelita USA Inc. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) ethylenediamine dihydrochloride, and *o*-phenylenediamine dihydrochloride were purchased from Thermo Fisher Scientific. 2,2'-azobisisobutyronitrile (AIBN) was purchased from Polysciences, Inc.. Sodium periodate (NaIO₄), 1,2-O-isopropylidene- α -D-glucofuranose, and methanol were obtained from Sigma-Aldrich. 2-hydroxyethylmethacrylate and phosphorus oxychloride were from Fluka. Triethylamine was purchased from Acros. Toluene, diethyl ether, tetrahydrofuran and anhydrous Na₂SO₄ were purchased from Mallinckrodt.

Synthesis of 2-(methacryloyloxy)ethyl phosphate (MOEP)

Phosphorus oxychloride (16.8g, 110 mmol) was added under argon to a stirred solution of 2-hydroxyethyl methacrylate (12g, 92 mmol) in toluene (340 ml). The reaction mixture was cooled to 0°C and triethylamine (39 ml, 276 mmol) was added. The reaction proceeded at 0°C for 30 mins, then at room temperature for 6 hrs. The white solid precipitate was recovered by filtration. Water (240 ml) was added to the filtrate and stirred overnight. The two layers were separated and the aqueous phase acidified, then extracted with THF: Ether (1:2, 6×225 ml). The organic phases were combined, dried over Na₂SO₄, and solvent evaporated to obtain the product as a pale yellow oil (67%, 12.2g). ¹H NMR spectroscopy (300 MHz, D₂O) δ 1.7 (3H, s), 4.0 (2H, m, POCH₂), 4.2 (2H, m, POCH₂CH₂), 5.5 (1H, s), 6.0 (1H, s); ¹³C NMR (75 MHz, D₂O) δ 17.4, 64.2 (d, ²J_{POC} = 8.3 Hz), 64.4 (d, ³J_{POCC} = 5.5 Hz), 127.2, 135.6, 169.4; ³¹P NMR (120 MHz, D₂O) δ 0.97 (s).

Poly(phosphodopamine) Synthesis

The *o*-DHP monomer (dopamine methacrylamide, DMA) was synthesized following a previously published method.[19] Briefly, a borate-dopamine complex was reacted with methacryloyl chloride at pH above 9. After reaction, the pH of the reaction mixture was reduced to less than 2 to dissociate the borate-catechol complex. The product was extracted with ethyl acetate and recrystallized from hexane.

Poly(phosphodopamine) (fig. 2) was synthesized as previously described [10] by free radical polymerization of MOEP and dopamine methacrylamide (DMA) initiated with AIBN in methanol. The polymerization proceeded at 60°C for 24 hrs. The copolymer was precipitated by addition of acetone. The precipitate was washed twice with acetone to remove residual monomers. The polymer was then dissolved in water and ultra-filtered on Pellicon Ultracel membranes (Millipore) with MWCO 1000 kDa followed by filtration with MWCO of 5 kDa. The concentration of phosphate and *o*-DHP sidechains were determined by NMR and UV/vis spectroscopy. The MW of the copolymer was determined by size exclusion chromatography on an AKTA FPLC system with a Superose 6 HR 10/300 column (GE Healthcare) in 0.05 M phosphate and 0.15 M NaCl (pH 7.4) calibrated with (poly)methacrylate standards. The (poly)phosphodopamine used in the experiments contained 17.7 mol% dopa side chains and the M_n was 20.6 kDa with PDI 2.42.

Gelatin Modification

Gelatin was amine-modified following the previously published method [11]. Briefly, gelatin (100 mg/ml) was mixed with ethylenediamine dihydrochloride and the pH adjusted to 5.2 with 0.1 M NaOH. EDC was added and the reaction was stirred at room temperature for 2 hrs. The modified gelatin was dialyzed against DI water. The concentration of primary amine sidechains was determined using ninhydrin assay with glycine as a standard. The amine-modified gelatin contained ~16 mol% primary amine groups and had a pI of 10.4.

Ninhydrin Assay

The ninhydrin stock solution was prepared by dissolving 1 g ninhydrin and 0.17 g hydrindantine in 10 mL 2-methoxyethanol and 12.4 mL of 4 N sodium acetate buffer (pH 5.5). 125 µL of freshly prepared ninhydrin solution was added to 250 µL gelatin solution

(0.2 mg/ml in 4 N sodium acetate buffer (pH 5.5)). The mixed solutions were incubated at 100°C for 15 minutes. After the samples were cooled to room temperature, 375 μ L 50% (v/v) ethanol in water was added to each sample. The content of primary amine was determined from UV absorption at 570 nm. Glycine (0–200 nmol/ml) was used as a standard.

Preparation of Gelatin Adhesive Complex Coacervate

A 50 mg/ml aqueous solution (pH 7.4) of amine-modified gelatin was added dropwise while stirring to a 50 mg/ml aqueous solution (pH 7.4) of (poly)phosphodopamine containing a molar ratio of 0.2 Ca^{2+} to phosphate sidechains. The mixture was stirred for 30 mins as the complex coacervate formed. The coacervated phase was collected by brief centrifugation. The periodate complexes were formed by dissolving 1, 2-O-isopropylidene- α -D-glucofuranose (IPGF) in DI water. Various ratios of NaIO_4 were added to the IPGF solution to form clear IPGF complexed periodate solutions. A white precipitate began to form in the periodate-IPGF solution after two hours. The IPGF/periodate solution was adjusted to pH 7.4. The IPGF complexed periodate was added to the complex coacervates at a molar ratio of 0.5 periodate to *o*-DHP sidechains and vortexed for thorough mixing before application to Al adherends.

IPGF/ NaIO_4 Complex Oxidation Kinetics

Due to secondary reactions of *o*-DHP after oxidation with NaIO_4 , *o*-phenylenediamine dihydrochloride (OPD) was used as a colorimetric substrate to investigate the kinetics of NaIO_4 /IPGF complex oxidation. An OPD stock solution (0.002 M, pH 7.4) was prepared in DI water. The OPD was aliquoted (125 μ L) into a 96 well plate. IPGF was mixed with NaIO_4 at ratios of 0:1, 0.5:1, 1:1 and 2:1. Solutions (125 μ L) of the IPGF/ NaIO_4 complex (pH 7.4) at a fixed NaIO_4 concentration of 0.001 M (a fixed 1:1 molar ratio to OPD) were added to the microplate wells with a multichannel pipette. The solution was mixed and absorbance at 450 nm was recorded over time.

Dynamic Rheology

The gelation kinetics of the adhesive complex coacervates oxidized with ratios of 0:1, 0.2:1, 1:1 and 2:1 IPGF/periodate were determined on a stress-controlled rheometer (TA Instruments, AR 2000) with a cone and plate configuration (20 mm diameter, 4° cone). Oscillatory time sweeps at a constant frequency of 1 Hz and a constant oscillatory strain of 1% were performed to track the elastic (G') and viscous moduli (G'') over time at 20°C. For each composition, the rheological experiments were conducted in triplicate.

Adhesive Bond Test

Adhesive bond strengths were measured with polished aluminum adherends in a standard lap shear configuration. Aluminum strips (6×50 mm) were cut from 5052 aluminum sheets with a water saw. The adherends were polished with 600 grit super fine sandpaper and then cleaned following ASTM D2651. The adherends were bonded within 12 hr of cleaning. Nine bonded samples were tested for each adhesive composition. Immediately after adding the IPTG/periodate complex the adhesive complex coacervates (6 μ L) were applied to wet

adherends, which were pressed together with an ~20 mm overlap. The bonded specimens were clamped with stainless steel clips and incubated fully submerged in water (pH 7.4) at 37°C for 24 hrs. The shear force at failure was determined on an Instron 3342 materials testing system (Instron corp, Canton MA) using a 100 N load cell at a strain rate of 1.2 mm min⁻¹. The aluminum adherends were fully submerged in a temperature-controlled water bath at 37°C during the test (Fig. 3). Post-failure specimens were analyzed by digital microscopy to determine bond areas and failure mode. Student's t-test was used to compare the means of the mechanical test results. P<0.05 was considered significant.

RESULTS

NMR spectroscopy

Formation of a stable complex between periodate and IPGF was confirmed by NMR spectroscopy (Fig. 1). As previously assigned and described [18], the signals from the 3-, 5-, and 6- protons of IPGF were shifted downfield in the presence of 1 molar equivalent of Na(IO₄). These protons are directly attached to the three carbons with free hydroxyl groups indicative of tridentate coordination of periodate by IPGF (Fig. 1B). The spectrum was unchanged after 24 hrs (not shown), which demonstrated that IPGF was not appreciably oxidized by periodate.

Oxidation Kinetics

Determining the rate and extent of periodate oxidation of *o*-DHP at neutral pH is complicated because the initial *ortho*-quinone product with a peak at 390 nm rapidly undergoes secondary reactions that turn the solution reddish brown with general absorption across the entire UV/visible spectrum. Therefore, the effect of IPGF complexation on the rate of periodate oxidation was investigated with *o*-phenylenediamine dihydrochloride (OPD), a compound commonly used as a peroxidase substrate that yields a stable, soluble, yellow-orange product ($A_{\max}$ 450 nm) when oxidized. Addition of 1.0 equivalent of free periodate relative to OPD resulted in complete oxidation of OPD within 3 mins (Fig. 3). When 0.5 equivalents of IPGF was added together with 1.0 equivalent of periodate the initial oxidation was rapid but the reaction took 40 mins to reach completion, which further demonstrated that periodate was not consumed by oxidizing the IPGF. The oxidation reaction was progressively slower with higher ratios of IPGF to periodate: the reaction was 83 and 52% complete after 40 mins with 1:1 and 2:1 ratios, respectively.

Rheology

The effect of complexing periodate with IPGF on the rate of adhesive complex coacervate curing was investigated by dynamic oscillatory rheology using a previously reported polyphosphate/polyaminated gelatin coacervate [11]. The molar ratios of amine sidechains and Ca²⁺ to phosphate sidechains were 1.0 and 0.2, respectively. The elastic (*G'*) and viscous (*G''*) moduli were recorded over time after addition of periodate at a fixed molar ratio of 0.5 relative to *o*-DHP in the coacervate. The ratio of IPGF to periodate was varied to evaluate the effect on coacervate crosslinking kinetics (Fig. 4). With 0 and 0.5 molar ratios of IPGF to periodate the crossover time between *G'* and *G''*, taken as a reference point for the transition point from a viscous fluid to a crosslinked elastic network [20], occurred in

less than the time it took to place the samples on the rheometer and begin measurements (less than one minute). With 1:1 and 2:1 molar ratios of IPGF to periodate the rate of crosslinking was substantially slower with crossover times were 9 ± 1.1 and 17 ± 1.9 mins, respectively. The decreased rate of crosslinking in the adhesive complex coacervate was also evident in the slowed increase in G' with higher ratios of IPGF to periodate.

Mechanical Shear Test

The adhesive properties of gelatin complex coacervates were evaluated in a standardized lap shear mechanical test using wet polished aluminum adherends. The bonded adherends were cured for 24 hr and bond strengths determined while fully submerged in a water bath at 37°C (Fig. 2). Bonds cured with uncomplexed periodate had a mean shear strength of 495 ± 159 kPa compared to 580 ± 88 kPa for bonds cured with a 1:1 complex of IPGF and periodate (Fig. 4). The mean bond strength of adhesive cured with complexed periodate was nearly 20% higher ($p < 0.005$) and had much lower variance than adhesive cured with uncomplexed periodate. In both cases adhesive remained on both surfaces of the aluminum adherends indicating the bond failed cohesively.

DISCUSSION

Stabilizing periodate in a sugar complex with three appropriately configured hydroxyl groups allows the initial cure rate and time to full cure to be controlled. By varying the ratio of sugar to periodate, the cure time can be adjusted over a wide range, from seconds to hours (Figs. 2 and 3). Moreover, biphasic curing profiles can be designed with substoichiometric ratios of sugar to periodate wherein a rapid partial cure is followed by a slower phase of curing. For some clinical applications of tissue adhesives or sealants based on crosslinking through periodate oxidized vicinal diols a mechanism to control the rate of oxidative crosslinking rate may be beneficial. This is particularly true for systems based on oxidative crosslinking through α -DHP functional groups [8–11, 21].

To begin with, stabilizing the periodate initiator allows it to be more thoroughly mixed with the α -DHP polymer(s) before initiation, which results in more homogeneous crosslinking. Without control, rapid crosslinking during mixing results in inhomogeneous crosslinking and a weaker sealant or adhesive. This is evident in the adhesive complex coacervate bonding data; stabilization of the periodate initiator resulted in less variance and greater bond strengths than uncontrolled crosslinking (Fig. 4). As a practical issue, the ability to control the curing reaction will also facilitate design of applicators that do not clog prematurely during adhesive mixing and delivery. A second, related benefit of controlled curing is that it allows time for positioning of the adherends after mixing and delivery. In the specific case of adhesive complex coacervates being developed for hard tissue repair controlled curing allows accurate positioning of small bone fragments before the adhesive sets [14].

It is generally accepted that the α -DHP form of dopa can promote interfacial adhesion while the oxidized quinone form is less adhesive [22, 23]. From this, it follows that oxidation of pendant α -DHP groups to crosslink a sealant or adhesive works against interfacial adhesion by the α -DHP groups; the more rapid the oxidation during delivery the fewer the α -DHP

groups available to adhere at the interface. This points to another benefit of controlled periodate curing; slowing the oxidation rate increases the number of α -DHP groups available to bind at the adherend interfaces and once bound the α -DHP groups are protected from oxidation. The α -DHP groups can thereby effectively contribute to both adhesion and cohesion of the material.

For the same purpose and benefits, organisms that secrete underwater adhesives containing dopa employ controlled cure strategies. These organisms must prevent premature hardening of their adhesives within the secretory system and yet efficiently trigger curing soon after secretion. Mussels may control the curing reaction by co-secreting a catechol oxidase that oxidizes dopa to dopaquinone to initiate crosslinking of adhesive plaque proteins [24]. The Sandcastle worm adhesive sets within 30 s following secretion but dopa-mediated covalent curing takes several hours as evident by the slow browning of the adhesive [25]. The mechanism that drives dopa crosslinking may be as simple as a pH jump when the adhesive is secreted into seawater [26]. At the pH of seawater (8.2) and in the presence of molecular oxygen α -DHP spontaneously oxidizes into the reactive quinone that undergoes covalent crosslinking. Controlled oxidation of dopa may allow the marine organisms to exploit dopa for both interfacial adhesion and covalent crosslinking. Indeed, the several mol% of dopa residues present in the fully cured adhesives are protected from oxidation, perhaps in coordination complexes with mineral surfaces [27, 28].

CONCLUSION

The rate of periodate oxidation of α -DHP and subsequent cross-linking reaction can be controlled by sequestering periodate in a reversible tridentate complex with carbohydrates containing three hydroxyl groups in the correct configuration. By varying the ratio of carbohydrate to periodate the curing rate could be varied over a wide range. The method offers three advantages: better mixing of adhesive and periodate initiator leads to more consistent and stronger bonds, the delay in curing allows time for accurate alignment of adherends, and interfacial adhesion of the α -DHP functionality is preserved during application.

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Figure 1.
NMR spectra and structures. **A)** 1,2-O-isopropylidene-α-D-glucofuranose. **B)** 1,2-O-isopropylidene-α-D-glucofuranose complexed with 1 molar equivalent of sodium periodate.

Figure 2.
Structures of polymers used to form complex coacervates. **A)** (Poly)phosphodopamine. **B)** Aminated non-gelling gelatin.

Figure 3.
Experimental set-up for underwater bond testing.

Figure 4.
Oxidation of OPD by various ratios of IPGF/periodate.

Figure 5.

Elastic and viscous moduli of adhesive oxidized by IPGF/Periodate complex. The *o*-DHP to periodate ratio was 2. The comparative measurements were made with constant strain of 0.1% and frequency of 1.0 Hz. G' = filled symbols. G'' = open symbols.

Figure 6.

Lap shear strength of adhesive complex coacervates oxidized with free periodate and periodate complexed with IPGF. Molar ratios: IPGF/Periodate=1, Periodate/*o*-DHP = 0.5

Scheme 1.

Kinetic control of *o*-DHP oxidation by complexing periodate.