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PEROXIDASE-CATALYZED CROSSLINKING OF FUNCTIONALIZED POLYASPARTIC ACID POLYMERS

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ABSTRACT

The formation of biodegradable hydrogels is reported based on enzymatic crosslinking reactions of modified polyaspartic acid. Poly(aspartic acid) polymers functionalized with aromatic groups were crosslinked in aqueous solution via peroxidase with hydrogen peroxide to form hydrogels. The reaction products were characterized based on time to gel, swelling ratio, sol fraction, storage and loss modulus, and entrapped enzyme. Optimum reaction conditions for gel synthesis were determined. Poly(aspartic acid) hydrogels have significant potential for use in biomedical applications such as in drug delivery due to the aqueous, nontoxic synthesis conditions as well as their inherent biodegradability by proteases.

Key Words: Peroxidase, Crosslinking; Hydrogel; Polyaspartic acid

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INTRODUCTION

Hydrogels are three dimensional polymeric networks synthesized using a variety of crosslinking methods, such as irradiation,[1] photoinitiators,[2] or other more specific chemistries.[3–5] A wide range of hydrogels have been synthesized and characterized. Hydrogels that can be prepared in an all-aqueous process using nontoxic chemicals, using ambient or mild reaction conditions, and that are biodegradable would be useful in biomedical applications. Gels prepared under these conditions and with these characteristics would extend hydrogel systems for incorporation of cells or labile chemicals, and avoid residual organic solvents or toxic organometallic compounds for biomedical applications, most notably for drug delivery.

To achieve these objectives, mimicking of biological cross-linking methods was explored through the use of enzymes.[6–10] Enzyme-based polymerization provides a useful option to consider in meeting the above goals since reactions can be carried out at ambient conditions in aqueous systems with direct control of the process through the modulation of enzyme activity.[11–13] In addition, if the starting polymers are biodegradable, as is the case with poly(aspartic acid),[14–16] and an enzymatic crosslinking process is utilized in the formation of the hydrogel, then the resulting crosslinked products should be biodegradable as previously demonstrated with other peroxidase-catalyzed cross-linked polymer systems.[17] Biodegradability, water solubility, and chemical properties have made poly(aspartic acid) a potentially useful polymer in detergents, personal care products, and water treatment, particularly when consideration for biodegradability is important.

In the present study, poly(aspartic acid) polymers functionalized with aromatic groups were crosslinked using peroxidase through initiation with hydrogen peroxide. The major goal of the present study was to establish a basis for enzyme catalyzed hydrogel formation of functionalized poly (aspartic acid) polymers under ambient conditions. This strategy is expected to lead to new families of biocompatible gel systems.

EXPERIMENTAL

Synthesis of Functionalized Polymers

The structural features and crosslinking reactions are illustrated in Fig. 1. In short, polysuccinimide (10,000 or 18,000 g/mol) was reacted with an aromatic functional group (tyrosine, tyramine, or aminophenol). Ring-opening of the succinimide initiated the coupling with the aromatic compound through an amide bond. Any remaining succinimide was ring-opened by treatment with NaOH. Control of degree of substitution (DS, fraction of monomer groups with an attached aromatic compound) was achieved by varying the molar ratio of the organic aromatic to succinimide in the reac-

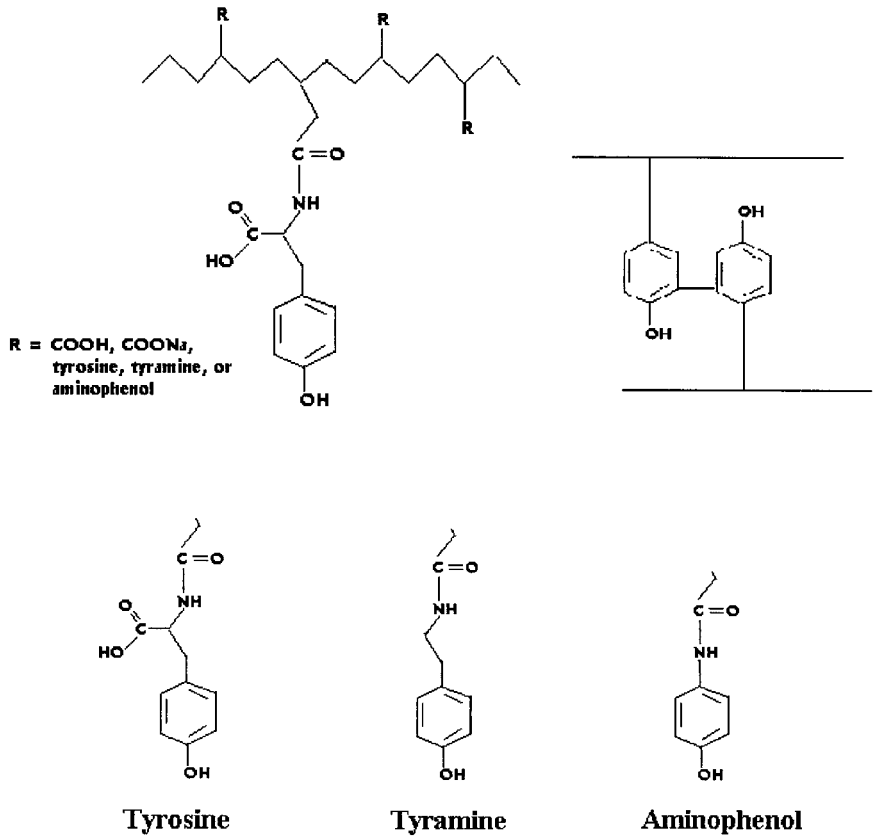


Figure 1. Schematic of the polyaspartic acid polymer and the tyrosine, tyramine, and aminophenol aromatic side groups.

tion. The molecular weight of the functionalized polymers varied from the backbone polyaspartamide weights depending on the degree of substitution with the aromatic functional groups. The DS of the functionalized polymers were 0.03, 0.1, 0.27, 0.34 for tyrosine (MW 18,000 of starting polysuccinimide), 0.1, 0.15, 0.25 for aminophenol (both 10,000 and 18,000 of starting succinimide), and 0.14, 0.26, 0.56, 0.66, 0.74, 0.92 for tyramine polymers (MW 18,000 of starting succinimide, except for DS 0.66 and 0.9, which were also 10,000).

Enzyme Reactivity and Synthesis of Gels

A range of reaction conditions was evaluated to determine enzyme reactivity in order to optimize the synthesis of the functionalized polyaspart amide gels. These variables were then explored in more detail to determine their effects on gel properties. *Buffer*—Borate buffer, potassium phosphate

buffer, Britton-Robinson buffer, and water were evaluated for their effect on peroxidase reactivity related to gel formation. All buffers were used at a concentration of 0.1 M with the pH adjusted to 6.5 unless otherwise noted. All buffer components were purchased from Aldrich. Milli-Q water with a resistance of 18 Ohm-cm was used (Millipore Corp.). A pH range from 5.5 to 8.5 was used in studying enzyme reactivity in gel formation and these pH studies were conducted using the Britton-Robinson buffer. Since fluorescence is dependent on pH, all solutions were titrated to pH 7 before measurements.

Enzymes

Seven peroxidases were evaluated for their reactivity with the various functionalized polymers: horseradish peroxidase (HRP) type I and II, lactoperoxidase (LPO), myeloperoxidase (MPO), soybean peroxidase (SPO), peroxidase from *Arthromyses ramosus* (APO), and sp502. All enzymes were purchased from Sigma chemical with the exception of sp502, which was a gift from Novo Nordisk (Denmark). In addition, two laccases were evaluated for their reactivity with the functionalized polymers, sp525 and sp804, both of which were a gift from Novo Nordisk. In the enzyme reactivity studies, the peroxidase concentration was 0.1 mg/mL. For the gel synthesis reactions, the concentration of enzyme was varied from 0.1 to 5 mg/mL.

Polymer Concentration

For the gel synthesis reactions, polymer concentration varied from 20–150 mg/mL (2–15 wt%).

Hydrogen Peroxide

The molar ratio of peroxide to aromatic side group (M/M) was varied from 0.25 to 1.00. The hydrogen peroxide used was a 30% w/v solution purchased from Aldrich which was diluted with purified water (Milli-Q water purification system, Millipore Corp, Bedford, MA) as necessary.

Temperature

The reaction temperature for gel synthesis was varied from 0 to 45°C. For these studies, all solutions were equilibrated at the reaction temperature

before they were mixed and reacted. The reacting solution was then maintained at that temperature.

Gel Synthesis

Reactions were run by first dissolving the functionalized polymer in buffer. The enzyme was added, mixed and then hydrogen peroxide was added. This solution was mixed several seconds and then left undisturbed for the remainder of the reaction. If a gelation reaction was agitated then a gel did not form. The total reaction volume was generally 300 μL. Several gel synthesis reactions were also run under nitrogen to evaluate the effect of molecular oxygen on enzyme reactivity.

Characterization of Reactions

The crosslink sites generated during synthesis could be used to monitor the reaction due to the fluorescence signal. A Shimadzu Model RF551 spectrofluorometric detector, or a Photon Technology International (PTI) Model A1010 fluorescence spectrometer with Felix® software were used. Reaction volumes were 3 mL for these types of studies and the excitation and emission wavelengths were 320 and 405 nm for tyrosine, 310 and 400 nm for tyramine, and 305 and 445 nm for aminophenol polymers, respectively. For kinetic studies, measurements were started immediately after the addition of hydrogen peroxide. For enzyme reactivity studies, the reacting solutions were kept in the dark and the fluorescence of solutions was measured 24 h after the addition of peroxide, when the reaction was complete.

CHARACTERIZATION OF POLYMERS AND GELS

Molecular Weight

Gel permeation chromatography (GPC) was performed on a Waters 2690 Alliance instrument using Millennium® software. Differential refractive index measurements were conducted using a Waters 410 instrument. The analytical column was a Shodex OHpak KB803 with guard column. Mobile phase was an aqueous solution of 0.05 M NaCl, 0.02 wt % NaN_3, pH 7.4, filtered through a 0.22 μm filter. Sample volumes of 50 μL were injected.

Gel Swelling

The swelling of hydrogels was characterized as a function of reaction conditions, including variables of pH and temperature. For the pH studies, a

0.1 M Britton-Robinson buffer was used for all pH conditions. Gels were placed in one liter of the appropriate pH solution with very gentle stirring and allowed to equilibrate for 24–48 h. Gel weights were taken several times to ensure that the gel had reached equilibrium. The gel was then removed and weighed, the solution pH lowered, and the gel was placed back in the solution at the new pH. After all weights were measured at each pH, the gel was then equilibrated in Milli-Q water for 2–3 days at a specific pH before it was dried and its dry weight obtained. For the temperature studies, the gels were first equilibrated in Milli-Q water for several days at room temperature before being placed at the appropriate temperature for equilibration for 5 days. The swelled weight of the gel was measured, the gel was dried, and the dry weight measured. For swelling as a function of synthesis conditions, the gels were equilibrated over 7 days in Milli-Q water before the swelled weight and dried weight were measured. All swelling solutions contained 0.02% sodium azide as bacteriostat. The swelling ratio was calculated as the weight of the swelled gel over the weight of the dried gel.

Temperature Stability

Gel stability was determined by equilibration in water for two days at room temperature before being placed in a hybridizing oven at a given temperature. The gels were maintained in the oven for up to two weeks, with the appearance and swelling of the gel checked each day. A temperature range from 23°C to 56°C was studied. Studies were carried out with and without the use of 0.02% sodium azide in the swelling solution.

Sol Fraction Analysis

Gels were placed in 10 mL of water with 0.02% sodium azide. After 7 days, an aliquot of swelling solution was removed and the absorbance measured at 280 nm When correlated to a calibration curve for the polymer, the concentration (and thus the mass) of unincorporated polymer in the swelling solution was determined. Absorbances of several samples in each experiment were checked on days 6, 7, and 8 and found to be the same, therefore samples had reached equilibrium by day 7. Absorbance measurements were made using a Hewlett-Packard 8452 UV/VIS spectrometer using quartz cuvettes.

Entrapped Enzyme

A qualitative determination of entrapped horseradish peroxidase (HRP) in the gel was performed in several ways. The determination of

active HRP was achieved by placing a piece of gel in buffer solution to which was added $\sim$10 μL of phenol (Aldrich) and 10 μL of peroxide. If active enzyme was present, the solution and/or gel turned brown. To qualitatively detect if HRP was released from a swelled gel into the swelling solution, an aliquot of swelling solution was placed in the UV/VIS spec and an absorbance spectrum taken. A peak at 405 nm indicated the presence of HRP, which in standard solutions can be detected to enzyme concentrations of $\sim$10 μg/mL.

DMA Measurements

Dynamic mechanical analysis (DMA) was done using an ARES parallel plate rheometer, with 25 mm plate diameter. One milliliter volume gels were synthesized in a disc shape with diameter 20 mm using an aluminum foil mold. The gels were removed from the mold and swelled in water for several days. The gel was then placed between the plates of the rheometer and the top plate moved to slightly compress the gel between the plates, the gap generally being $\sim$3 mm. Measurements of storage (G') and loss (G'') modulus were taken at a frequency range from 0.1 to 150 rad/s

Raman Spectroscopy and NMR

Raman spectroscopy of uncrosslinked dry polymer and crosslinked dried gel material was carried out using a Bruker Equinox 55 ATR-FTIR, with Raman spectroscopy. Proton NMR of uncrosslinked polymer and crosslinked gel material was performed using a Bruker NMR, 300 MHz.

RESULTS AND DISCUSSON

Reaction Parameters for Gel Synthesis

In order to gain an understanding of the enzymatic cross-linking of functionalized polyaspartic acid polymers, a range of reaction conditions was initially evaluated. This included varying polymer molecular weight, degree and nature of aromatic substitution, buffer, pH, and enzyme. In the second stage of the studies a more refined window of conditions was investigated for the formation of gels. Conditions included enzyme concentration, polymer concentration, peroxide concentration, and DS of the functional group on the polymer. The gels were then characterized in order to correlate reaction conditions with gel properties.

Polymer Molecular Weight

Two main chain molecular weights of the starting polysuccinimide polymer, 10,000 and 18,000 g/mol, were tested for gelation. Both tyramine-functionalized polymers (DS 0.66, 0.92) and aminophenol-functionalized polymers (DS 0.15, 0.2) from 10 K molecular weight polysuccinimide were reacted. At concentrations up to 10 wt% polymer in solution, in a pH range from 6.5 to 8.0, from 0.1 to 2 mg/mL HRP type II enzyme with a molar ratio 0.5–2.0 of peroxide to functional group, the solutions did not gel. Under the best conditions, the DS 0.92 tyramine polymer formed a very weak gel after 24 h. However, for the same DS polymers at 18 K molecular weight the solutions easily formed stable gels. It is likely that the lower molecular weight polysuccinimide was too short for sufficient molecular entanglement and intermolecular crosslinking to achieve gelation.[1,10] Based on these results, all subsequent studies on gel synthesis and characterization were carried out using functionalized 18 K g/mol polysuccinimide.

Reaction Buffer

Three buffers as well as purified water were tested for their effect on enzyme reactivity with tyrosine-functionalized polymers. Figure 2 shows the dityrosine fluorescence signal from these solutions with DS 0.27 tyrosine polymer, as well as the fluorescence from solutions of free L-tyrosine at an equivalent molar concentration of tyrosine as the functionalized polymer (1.1 mM). The greatest reactivity with the greatest fluorescence for both the polymer and free tyrosine was with the Britton-Robinson buffer. Similar results were also seen for the aminophenol-functionalized polymer when comparing reaction in phosphate buffer and Britton-Robinson buffer. Therefore, gel reactions were run in the Britton-Robinson buffer, which spans a wide range of pH, from pH 4–11.[18–19]

Peroxidase and Laccase

A number of different enzymes were reacted with the different functionalized polymers to study enzyme reactivity and gel formation. In general, the products of enzyme cross-linked aromatics are fluorophores.[20–22] The fluorescence signal was used as a qualitative measure of an enzyme's reactivity with the various functional groups on the polyaspartic acid polymers as an indication of crosslinking and gel formation at higher polymer concentrations. Reactivity of the enzymes was tested by measuring the fluorescence 24 h after initiation of the reaction with the addition of peroxide. Fig. 3a shows the fluorescence of six peroxidases reacted with DS

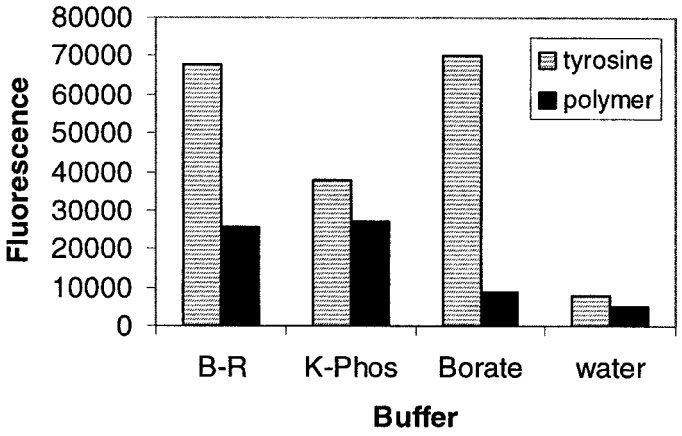


Figure 2. Fluorescence measurements from solutions of tyrosine polymer (DS 0.27) and free L-tyrosine having equivalent concentrations of tyrosine (1.1 mM, enzyme (HRP, 0.1 mg/mL), and peroxide (equimolar to tyrosine), reacted at pH 7.5 in different buffers (0.1 M), and in water.

0.27 tyrosine polymer at pH 7.5. HRP type II and APO are equally most reactive with the polymer, SPO exhibited slight reactivity, and the remaining enzymes showed little or no signal above the baseline. The GPC data in Fig. 4a show very similar peaks for the tyrosine polymer reacted with HRP and APO, in agreement with the fluorescence data. Also shown in Fig. 4 are the GPC data for tyramine polymer (DS 0.66, 10k) reacted with HRP, SPO, and LPO peroxidases. The peaks indicate decreasing enzyme reactivity in the order LPO > SPO > HRP, which agrees with the fluorescence data shown in Fig. 3b. Fluorescence data for reactivity with the aminophenol polymer (DS 0.15, 10 K are shown in Fig. 3c, which shows that there was no preference by any of the enzymes in reaction with this polymer.

The gelation experiments often did not correlate with the fluorescent data. The tyrosine polymer solutions never gelled despite varying conditions of pH, polymer concentration, enzyme concentration, or peroxide concentration. The aminophenol polymers only gelled when catalyzed with HRP type II or APO. The tyramine polymers formed gels when catalyzed with HRP types I and II, APO, and SPO; a wide range of conditions were tried with LPO and sp502 without gelation. A possible explanation for the discrepancy between fluorescence and gelation is that other linkages or oxidation products may be formed that are not fluorescent, such as through an ether or ketone linkage,[19,20,22,23] rather than just the C-C cross links which lead to a fluorescent product. Thus the fluorescence can be used as a qualitative indicator of one product of the reaction and not an indication of the overall reactivity of the enzyme with the substrates leading to gelation. It is also possible that reactivity of the enzymes is dependent on the

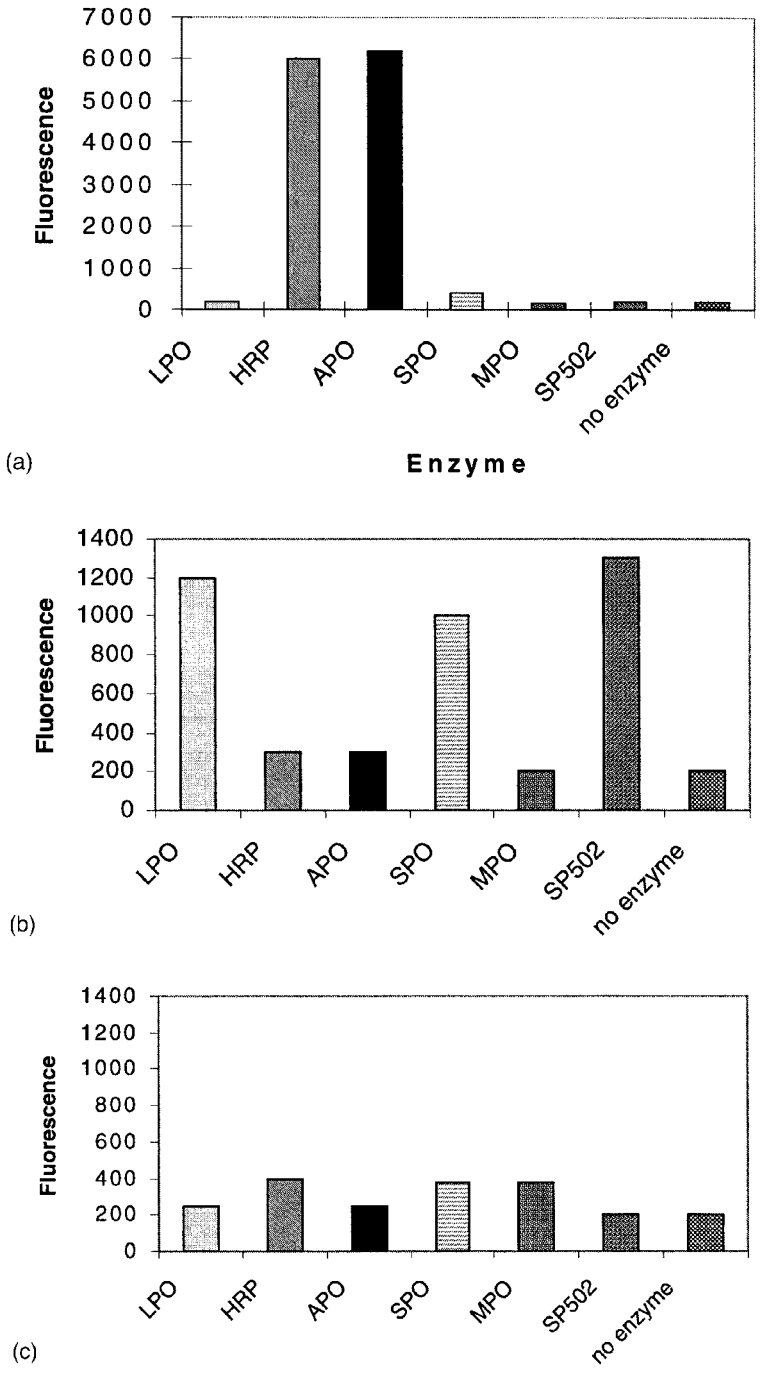
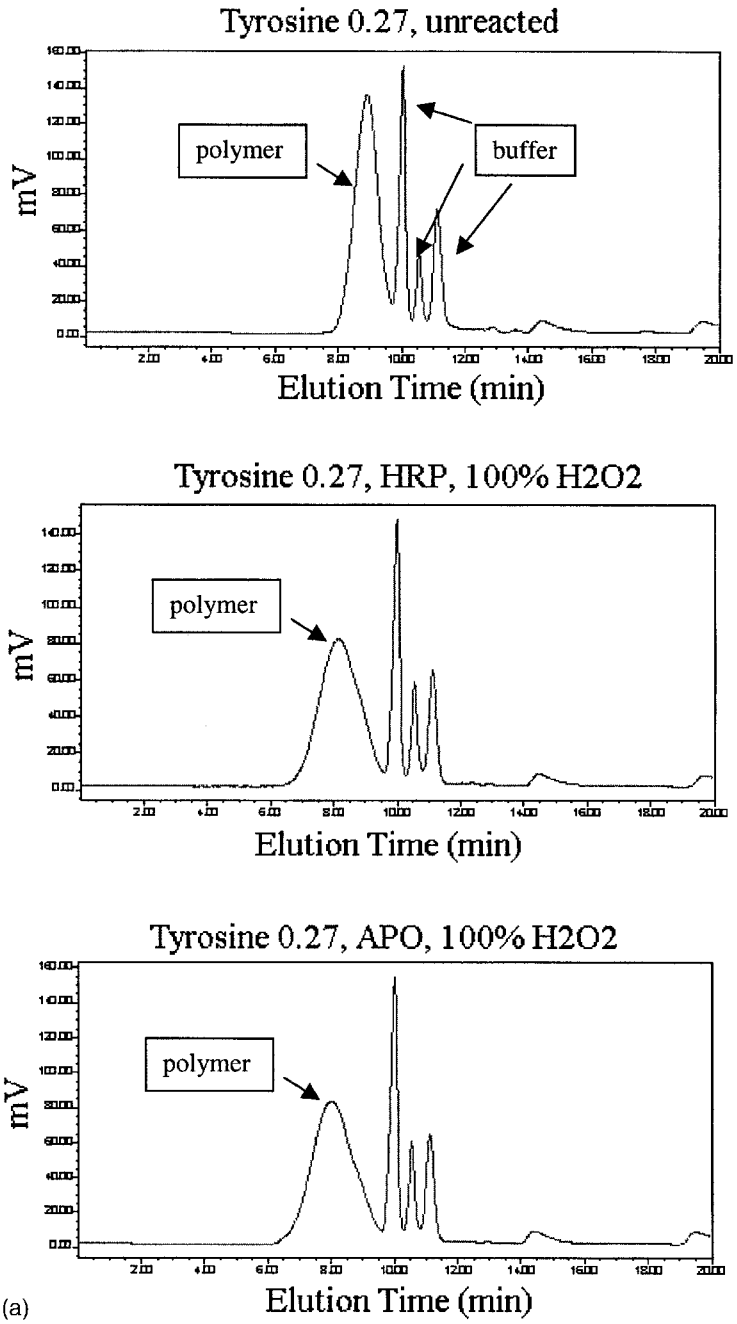


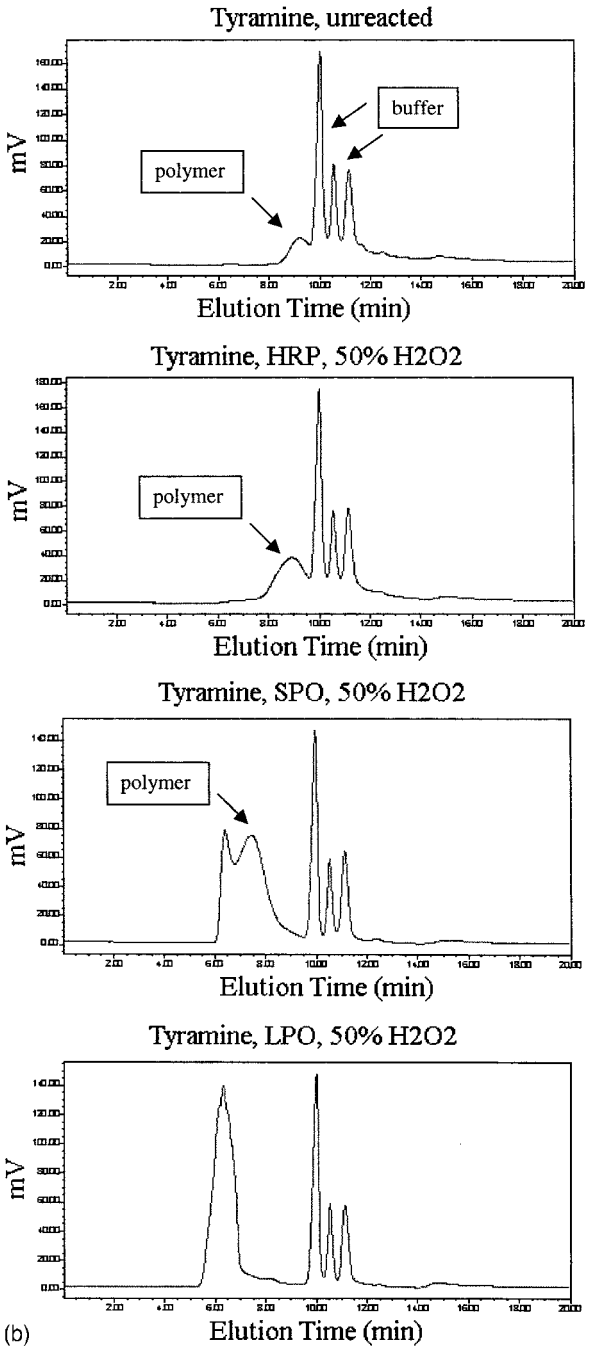
Figure 3. Fluorescence of functionalized polymer solutions reacted with different peroxidase enzymes: a) tyrosine polymer, DS 0.27; b) tyramine polymer, DS 0.66; c) aminophenol polymer, DS 0.15. (enzyme concentration 0.1 mg/mL, peroxide equimolar to aromatic functional group, 0.1 M borate buffer, pH 7.5).



(a)

Figure 4. GPC data from reacted solutions of a) tyrosine polymer, DS 0.27, with HRP and APO; and b) tyramine polymer, DS 0.66, with HRP, LPO, and SPO. Reaction conditions: 2 wt% polymer, 0.1 M Britton-Robinson buffer, pH 7.5, 0.5 mg/mL enzyme, 0.5 molar ratio of peroxide to functional group.

(*continued*)



(b)

Figure 4. Continued.

concentration of polymer in solution.[18] In general, both HRP type II and APO were the most reactive with the functionalized polyaspartamide polymers in terms of gel formation, despite their different spectroscopic behavior[24,25] and reactivity with various substrates.[19]

Neither laccase showed significant reactivity with any of the polymers. This was based on lack of fluorescence signal, as well as by no increase in solution viscosity and a lack of gel formation. In general, laccases are more reactive in a pH range from 4–6.[26]

Dependence on pH

From the literature, it has been found that optimum pH for reaction of HRP with aromatic compounds is in the range of pH 6–8.[18,20] The fluorescence of solutions reacted at four different pHs in the range 5.5 to 8.5 is shown in Fig. 5a. All three functionalized polymers show a mild dependence on pH in this range with slightly increasing reactivity with pH, in agreement with the literature for HRP activity. Fig. 5b shows the results for APO enzyme with aminophenol and tyramine polymers, showing similar results to HRP. However, in looking at gelation, from pH 6.25 to 8.5, it was found that gelation of tyramine polymer (DS 0.92, 18 K) with HRP was faster at the lower pHs (see Table 1), and that stronger gels were formed at the lower pHs. These results seem to indicate higher reactivity and greater crosslinking at the lower pHs not seen with fluorescence. Therefore, in the synthesis of gels for gel characterization, a pH of 6.5 was generally used.

Gel Synthesis

Tyrosine Polymers

Four degrees of substitution of tyrosine polymers (18 K starting polysuccinimide) were studied for gel formation: 0.03, 0.1, 0.27, and 0.34. The reactivity of these four polymers with HRP type II was very different. Figure 6 shows the GPC data from reacted solutions of the higher three DS polymers. The DS 0.27 showed the greatest reactivity, with a shift of the polymer peak to earlier elution times indicating larger molecular weight products. There was also significant peak broadening. The DS 0.34 shows slightly greater reactivity than DS 0.1, but in both these cases the initial polymer peak is unchanged (uncrosslinked), indicating little reaction. Reactions with APO, the other enzyme found to be most reactive with these polymers, showed almost identical results. When the DS 0.27 polymer was reacted to form a gel, no gelation occurred. Polymer concentrations up to 10 wt%, enzyme concentrations from 0.1 to 2 mg/mL, and added peroxide from 0.5 to 4 times the molar amount of tyrosine present, added in increments or

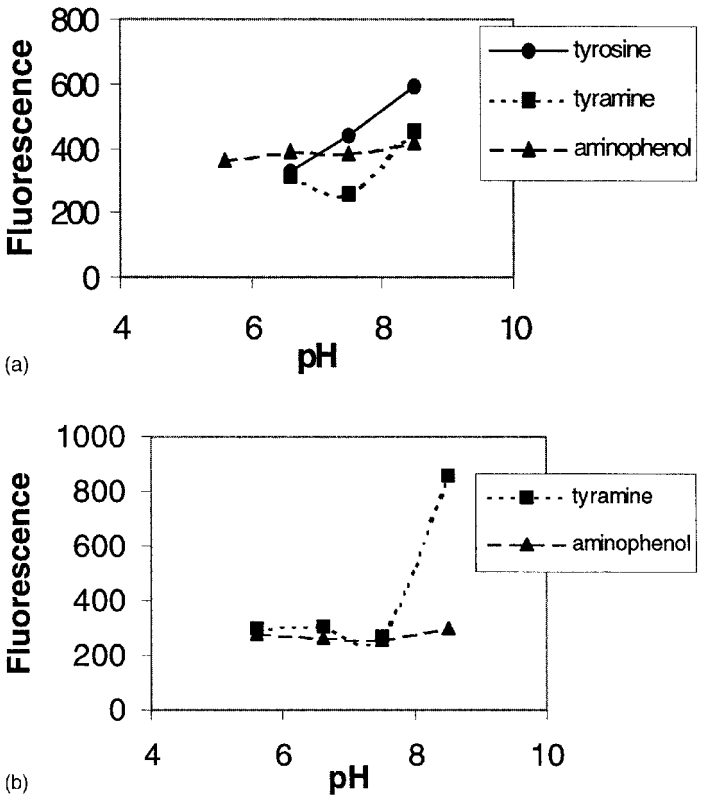


Figure 5. Fluorescence of reacted solutions as a function of the pH of the reaction using a) HRP Type II, and b) APO. Reaction conditions: 0.1 M Britton-Robinson buffer, pH 7.5, 0.1 mg/mL enzyme, 0.7 mg/mL polymer, peroxide 2× molar excess over moles of functional group. Tyrosine (DS 0.27), tyramine (DS 0.66), aminophenol (DS 0.15).

Table 1. Effect of Solution pH on Time to Gel (Tyramine Polymer, DS 0.92, 12 wt% Polymer, 50 mol% Peroxide as Tyramine, 2 mg/mL HRP)

Buffer pH	Gel Formed (Time in h)
6.25	+ + + (0.35)
6.50	+ + + (1)
7.14	+ + (24)
8.49	+ + (24)

+ + + strong gel.
+ + weak gel.

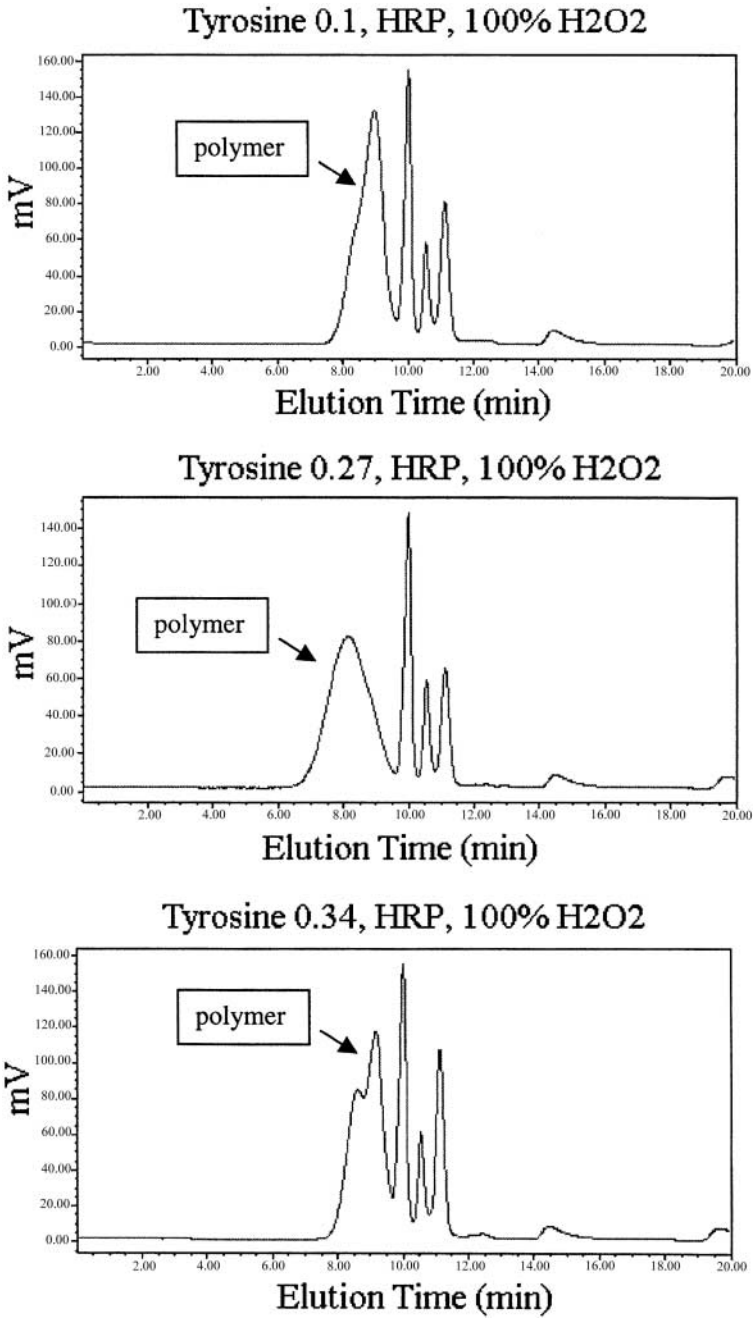


Figure 6. GPC chromatograms of reacted tyrosine polymer solutions, DS 0.1, 0.27, 0.34. Reaction conditions: 2 wt% polymer, 0.1 M Britton-Robinson buffer, pH 7.5, 0.5 mg/mL enzyme, 0.5 molar ratio of peroxide to functional group.

all at once, were evaluated. No visible change in solution viscosity occurred indicating little crosslinking. In order to gain some insight into the low reactivity of the tyrosine polymers, reactions of tyrosine polymer DS 0.27 were compared with free L-tyrosine in which the solutions had equivalent molar concentrations of tyrosine (1.1 mM). Figure 7 shows the fluorescence curves from these two solutions. The fluorescence from free tyrosine is nearly ten times that of the polymer. This difference in reactivity could be due to a number of factors.

The presence of the many acid groups from the polyaspartic acid main chain (those with no tyrosine attached) may cause an unfavorable interaction with the active site of the enzyme, either through steric hindrance or charge interactions. It had been found previously with tyrosine in peptides containing acid side chains (glu-tyr and glu-lys-tyr) that no crosslinking occurred with five different peroxidases.[19] However, evidence against this factor is found in the fact that the DS 0.34 polymer, which has less aspartic acid

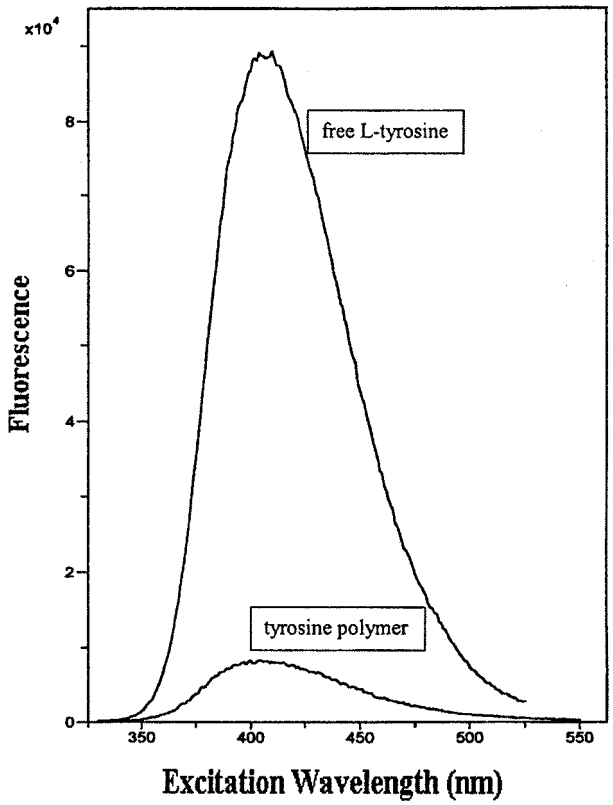


Figure 7. Fluorescence peak for reacted solutions of tyrosine polymer (DS 0.27) and free L-tyrosine. Reaction conditions: 0.1 M borate buffer, pH 7.5, 1.1 mM tyrosine, 0.1 mg/mL HRP, peroxide equimoler to tyrosine.

groups due to the higher DS, showed lower reactivity than the DS 0.27. In addition, aminophenol polymers of DS 0.25 readily formed gels in solution.

The polymer chain conformation in solution may effect crosslinking. Tyrosine is a relatively hydrophobic molecule with limited solubility in water. It is possible that at the larger DS values, the polymer chains fold such that the tyrosine molecules are shielded in the center of the molecule, increasing hydrophobic interactions, with the more hydrophilic acid groups facing outward into aqueous solution. This would prevent the enzyme from having access to the tyrosine groups, and thus prevent intermolecular crosslinking. However, tyramine also has limited solubility in aqueous solution and tyramine polymers readily form gels.

The most important factor effecting enzyme reactivity with the tyrosine polymers may be the free acid group on the tyrosine. When this acid group is not present, i.e., tyramine, the polymers are much more reactive and readily form gels in solution. As free tyrosine does not exhibit low reactivity, a possibility is that there is an electrostatic interaction between the acid groups on the main chain and that on the tyrosine side group causing a steric restriction on the tyrosine. Since the active site of HRP (and the other peroxidase enzymes) is a hydrophobic pocket that is sensitive to neighboring charges,[24,27,28] the nearby acid on tyrosine could interfere with the interaction of the aromatic with the active site. A similar occurrence was found with the crosslinking enzyme transglutaminase, which also has a hydrophobic pocket at the active site, where to prevent unfavorable interaction with the enzyme, the substrate glutaminamide was used rather than glutamine.[9]

Aminophenol Polymers

Several degrees of substitution of the aminophenol polymers (18 K starting polysuccinimide) were used in gel formation reactions. For DS values lower than 0.2 with the same range of conditions used for the tyrosine polymers, the polymers did not gel. However, at DS 0.25, the polymers readily formed gels within 15 min (or within 1–2 min with APO) from the initiation of the reaction with hydrogen peroxide. In general, conditions necessary for gel formation were: a) polymer concentration $\geq$2%, b) HRP or APO concentrations $\geq$0.5 mg/mL, and c) ratio of 0.25–1.00 of moles of peroxide added relative to moles of aminophenol in solution. Peroxide added all at once produced stronger gels than when added incrementally every few minutes. This is probably due to the fact that each added increment of peroxide required mixing of the solution, and it had been found that continued agitation of the gelling solutions was found to inhibit gelation. Control solutions containing polymer and peroxide without enzyme did not form a gel, nor was any fluorescence observed.

Tyramine Polymers

Since a DS > 0.25 of the aminophenol could not be successfully synthesized, polymers synthesized with tyramine (18 K were characterized since large quantities of this polymer could be synthesized with a wide range of DS for studies of gelation. The DS values included 0.14, 0.26, 0.56, 0.66, 0.74, 0.92. Only the lowest DS polymer did not form a gel while for the remainder of the polymers gelation was dependent on DS (Table 2). In general, gelation occurred less readily at DS values <0.5, went through a maximum between DS 0.5–0.7, and then declined again at DS 0.9. The explanations for this gelation behavior could be similar to that described for the tyrosine polymers, a possible need for a balance between aspartic acid electrostatic effects at the lower DS with tyramine hydrophobic effects at the higher DS.

Other Factors Effecting Gel Synthesis

Two other variables were briefly evaluated for their effect on gel synthesis, temperature and oxygen. Oxygen can have an inhibitory effect on some radical reactions, however, this was not the case with the gelation reactions. It was found that a solution of tyramine polymer (DS = 0.92) gelled at a similar rate in the presence of nitrogen or oxygen. Gel synthesis was dependent on temperature, increasing temperature from 23°C to 36°C led to the formation of weaker and stickier gels (Table 3). Therefore, enzyme reactivity and crosslinking did not increase with reaction temperature over this range.

Gel Characterization

Swelling vs. Solution pH

Figure 8 shows the dependence of swelling on solution pH for both aminophenol (DS = 0.25) and tyramine (DS = 0.92) polymers. Both show a constant swelling ratio at pH values greater than 6, with a marked drop in swelling below pH 6. This behavior is as expected, where the marked drop in swelling with pH is due reaching the pKa of the acid groups in the polymer.[10] With the acid groups no longer charged, there is no electrostatic repulsion and thus the gel shrinks. This behavior is seen with polyacrylic acid and polymethacrylic acid polymer gels.[29,30] This forms the basis of 'smart gel' behavior that can be utilized to control release of carried materials and rates of degradation.

Table 2. Gel Time of Tyramine Polymers Under Various Conditions; Standard Conditions: 7 wt% Polymer, 2 mg/mL HRP II, 50 mol% H_2O_2 Relative to Moles of Tyramine, Unless Otherwise Varied

Polymer Concentration		Gel Time (min)	
(wt%)	DS 0.56	DS 0.74	DS 0.92
4	35	5	No gel
6	–	–	600
7	1	1	–
8	–	–	600
10	1	1	45
12	1	1	45
15	–	–	5
Enzyme Concentration		Gel Time (min)	
(mg/mL)	DS 0.56	DS 0.74	DS 0.92
0.5	60	5	300
1	20	2	120
1.5	7	2	120
2	5	1	120
3	5	1	120
4	5	1	120
H_2O_2 Added		Gel Time (min)	
mol% H_2O_2 vs. Tyra	DS 0.56	DS 0.74	DS 0.92
25	1	1	No gel
35	1	1	20
45	5	1	30
60	10	1	90
75	15	3	100
100	15	4	100
Other DS Tyramine	Gel Time (min)		
0.14	No gel		
0.26	40		
0.66	10		

Swelling vs. Temperature

The dependence of swelling on temperature was tested with two aminophenol gels. The results in Fig. 9 show that there is little dependence on temperature from 4–23°C, and that swelling mildly increases at higher temperatures up to 46°C. Gels that were maintained at temperatures greater than 50°C degraded (lost gel consistency) after several days to two weeks possibly due to slow hydrolysis caused by residual azide in the gel.

Table 3. Gelation of Tyramine Polymer (DS 0.92) as a Function of Temperature

Temperature (°C)	Gel Formed (time in min)
0	+ + + (30)
10	+ + + (11)
26	+ + + (15)
30	+ + + (23)
32	+ + + (25)
36	+ + + (30)
40	+ + (120)
45	No gel (240)

+ + + strong gel.
+ + weak gel.

Swelling Dependence on Synthesis Conditions

The swelling of tyramine polymer gels synthesized from three different DS polymers (0.56, 0.74, 0.92) was studied as a function of polymer concentration, HRP concentration, and peroxide (Fig. 10). The gel synthesized from DS 0.5 polymer was the most highly crosslinked, showing the lowest swelling ratios. Gels synthesized with DS 0.5 and 0.74 polymer had the same overall behavior in their dependence on the three parameters, having little dependence on HRP concentration and only slight dependence on peroxide and polymer concentrations at the lower values. The DS 0.92 polymer gels show the greatest dependence on all three parameters. In addition, both the 0.56 and 0.74 DS gels tended to be firm and relatively easy to handle, whereas the gels synthesized with DS 0.92 polymer were the weakest gels and were very flexible and soft. The swelling ratio for the DS 0.92 gels was expected to be the highest for the three polymers because they formed the weakest gels (therefore, lowest crosslink density), but was found to be the lowest. This may be because at such a high DS, the concentration of aspartic acid residues in these gels is much lower than in the other polymers. This leads to less electrostatic repulsion, lower hydrophilicity, and thus lower swelling.

Sol Fraction

As a measure of the efficiency of the crosslinking reaction in these gels, the amount of unincorporated polymer was measured as a function of polymer concentration, enzyme concentration, and peroxide added for the

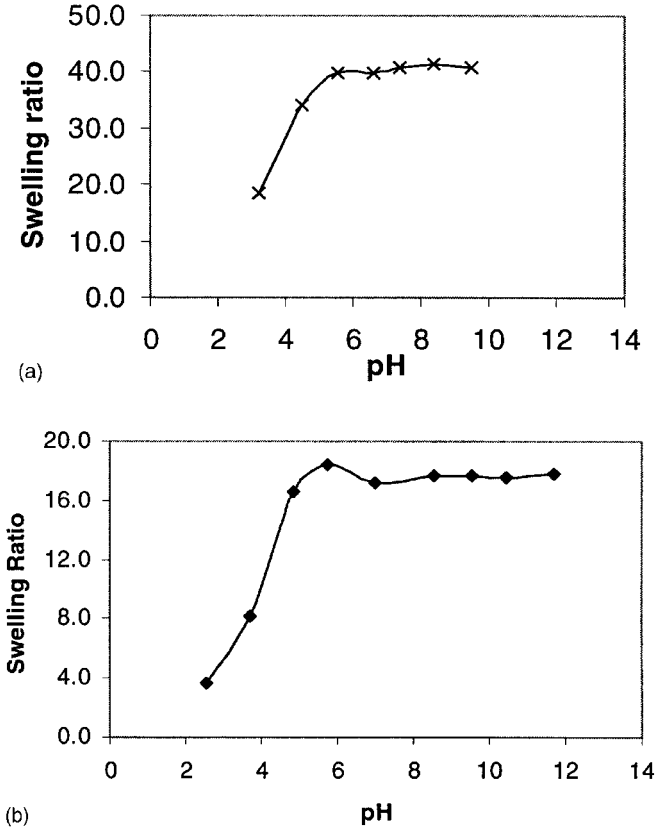


Figure 8. Swelling as a function of pH for a) aminophenol gel (5 wt% polymer, DS 0.25, 1 mg/mL HRP, peroxide equimolar to aminophenol), and b) tyramine gel (12 wt% polymer, DS 0.9, 2 mg/mL HRP, 0.5 molar ratio peroxide to tyramine).

same three DS tyramine polymers that were used in the swelling ratio studies (Fig. 11). As in the swelling studies, the DS 0.56 and 0.74 polymer gels show similar dependence on all three parameters, with 80–95% of the polymer being incorporated into the gel in most cases. The results for the DS 0.92 gels exemplify the inefficient crosslinking and thus weak structure of these gels, with $\leq$75% of the polymer covalently crosslinked into the gel. It is possible that steric hindrance due to closely surrounding tyrosine groups influences the interaction of the enzyme with the side groups, thus decreasing the crosslinking reaction. Neighboring groups on proteins and peptides are known to effect reactivity with the peroxidase enzymes.[19,21,28]

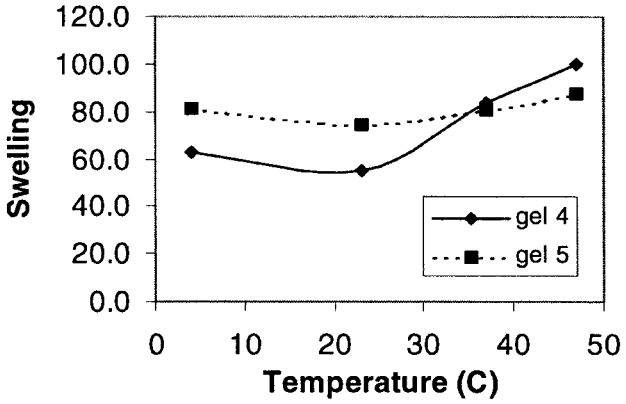


Figure 9. Swelling as a function of temperature for aminophenol gels (gel 1: 3 wt% polymer, 0.5 mg/mL HRP, peroxide equimolar to aminophenol; gel 2: 5 wt% polymer, 1 mg/mL HRP, peroxide equimolar to aminophenol).

DMA Analysis

Mechanical strength of the polyaspartamide gels was determined by dynamic mechanical analysis to correlate with gel synthesis conditions. DMA measurements yield values of storage (G′) and loss (G″) modulus which give a measure of the resistance and dynamic response of these gels to an ocillatory shear stress.[31] Figure 12a shows the influence of tyramine DS on gel strength, all other gel synthesis conditions being the same. The gel formed from DS 0.56 polymer exhibited the greatest strength and the DS 0.92 exhibits the weakest, which agree with the results found in the swelling and sol fraction studies. Again, these differences are most likely due to the difference in crosslink density in the gels, where a higher crosslink density increases the modulus of the material.[31] The effect of polymer concentration is shown in Fig. 12b for the gel synthesized from DS 0.74 polymer. As expected, increasing polymer concentration increases gel modulus. This is due to greater molecular entanglement leading to higher crosslinking at the higher polymer concentrations.[1,9,10] To see how these tyramine gels compare to other common gel materials, the modulus of a 10% tyramine polymer (DS 0.74) was evaluated with a 1% agarose gel and a 10% polyacrylamide gel, all of which were equilibrated in water before the measurements (Fig. 12c). The tyramine gel strength falls between the two other gels, with the agarose gel exhibiting the greatest strength of the three gels. Therefore the strength of the functionalized polyaspartic acid gels are comparable to common gel materials.

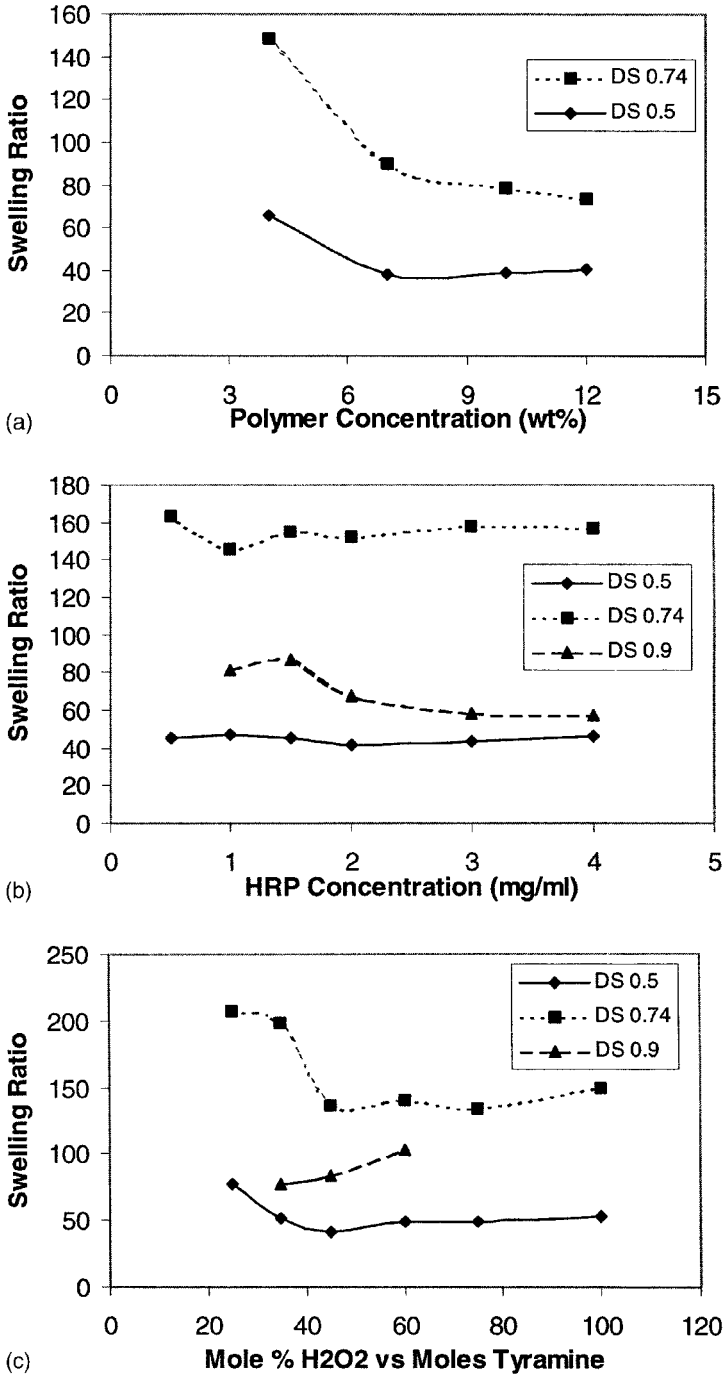


Figure 10. Swelling of tyramine polymer gels (DS 0.56, 0.74, 0.92) as a function of a) polymer concentration, b) enzyme concentration, and c) molar ratio peroxide to tyramine (synthesis conditions of unvaried parameters: 7 wt% polymer, 2 mg/mL HRP, 0.5 molar ratio of peroxide to tyramine, 0.1 M Britton-Robinson buffer, pH 6.5).

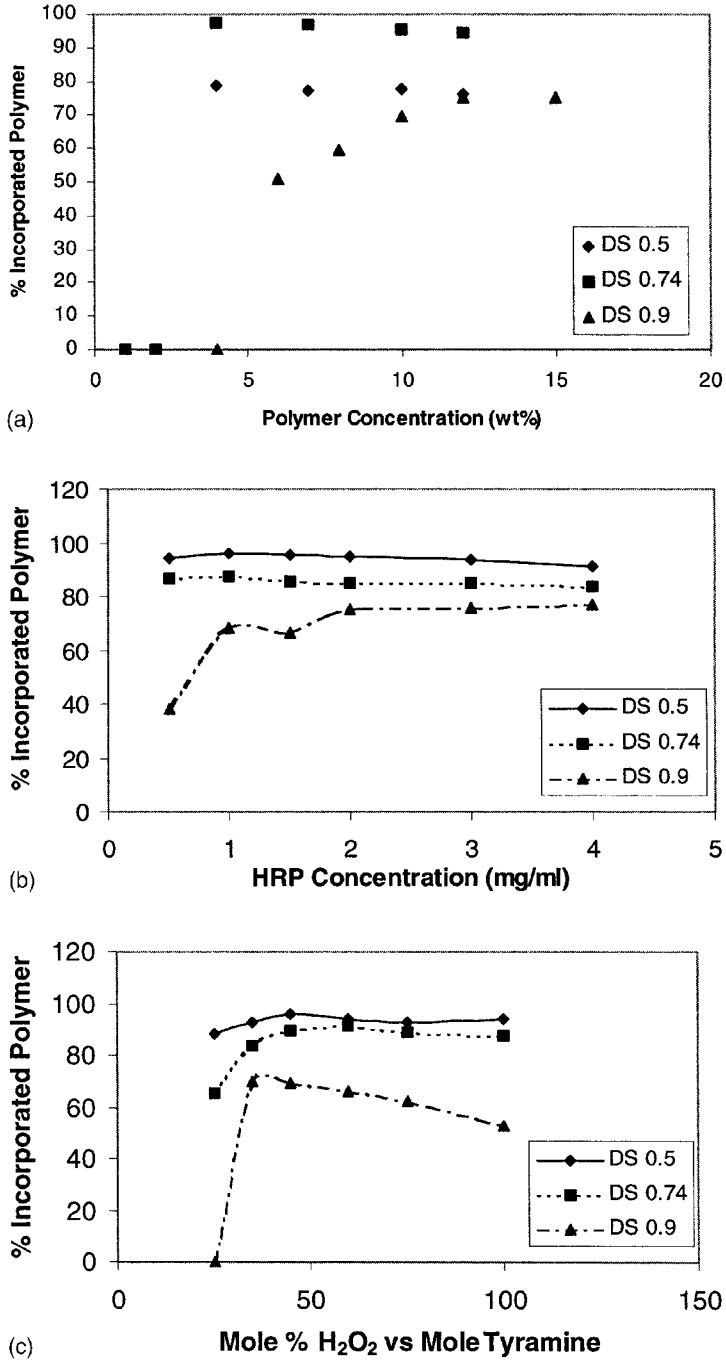


Figure 11. Fraction of incorporated tyramine polymer as a function of a) polymer concentration, b) enzyme concentration, and c) molar ratio peroxide to tyramine. (Synthesis conditions of unvaried parameters: 7 wt% polymer, 2 mg/mL HRP, 0.5 molar ratio of peroxide to tyramine, 0.1 M Britton-Robinson buffer, pH 6.5).

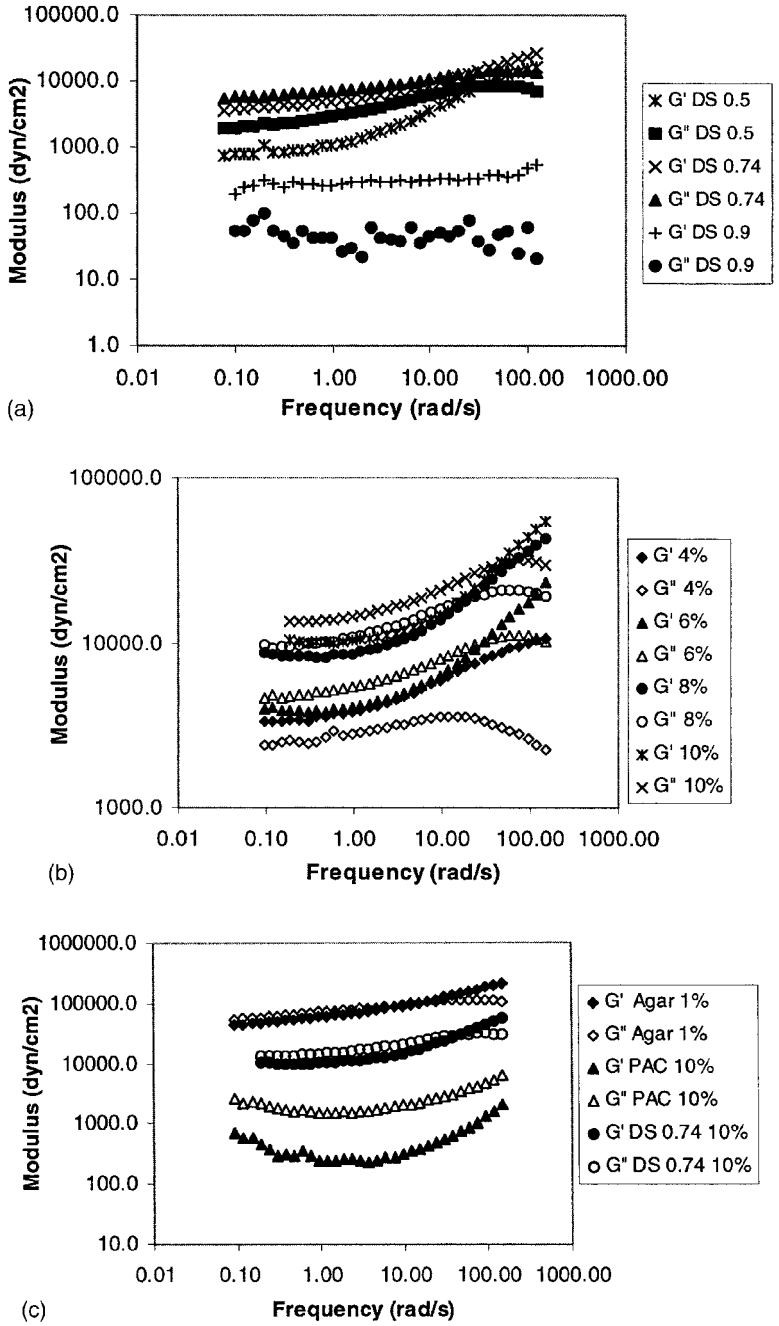


Figure 12. DMA data on tyramine polymer gels: a) effect of tyramine DS (7 wt% polymer); b) effect of polymer concentration (DS 0.74), c) comparison of gel strength to 1% agarose and 10% polyacrylamide gels (synthesis conditions of tyramine gels same as those of Figs. 10 and 11).

NMR and Raman Spectroscopy Analysis

Both NMR and Raman spectroscopy were used to try and elucidate the chemistry of the crosslinks in the gel materials. The primary link between the aromatic groups is expected to be a direct carbon-carbon bond, which leads to the fluorescent compounds.[22] However, C-O-C ether linkages could also be formed through the aromatic hydroxyl group, which is not a fluorophore. In numerous studies on tyrosine crosslinking in proteins and peptides, generally the proteins are hydrolysed and amino acid analysis is performed. If ether linkages are present they would be hydrolyzed in the acid hydrolysis and thus not detected in subsequent analysis. Figure 13 shows the ^{1}H-NMR results from untreated tyramine polymer as well as from a crosslinked polymer gel. In a focussed region of the scan from 6.2 to 7.4 ppm, the appearance of two small peaks, one at 6.7 ppm and one at 6.36 ppm, can be seen in the gel that are not present in the uncrosslinked polymer. These two peaks appear to be a shift downfield from the peaks at 6.58 and 6.88 ppm, where these peaks represent the protons in the aromatic ring. Therefore, there is a change in some of the aromatic protons, however, the exact chemistry of the crosslink cannot be determined. These same conclusions were drawn from the Raman spectroscopy results (Fig. 14). The largest difference in the scans appears at 840 cm^{-1} and 1225 cm^{-1}, which also indicates changes in the aromatic moiety. Previous studies with peroxidase catalyzed polymerization of aromatics have shown via FTIR and ^{13}C-NMR that the primary linkage between aromatics is *ortho-ortho* if that position is available on the ring.[23,32,33]

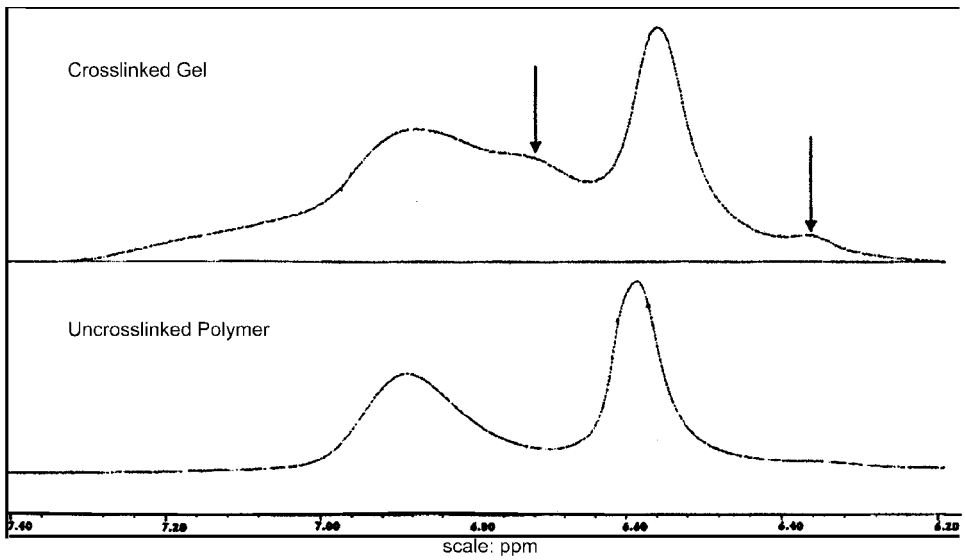


Figure 13. ^{1}H NMR results on unreacted tyramine polymer and crosslinked tyramine gel (DS 0.92).

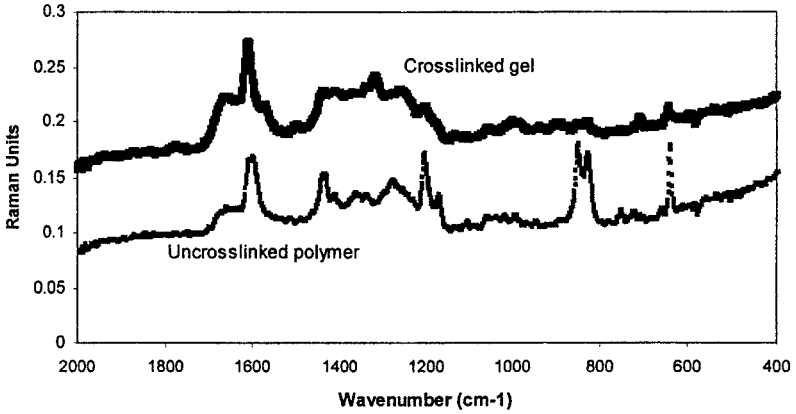


Figure 14. Raman spectroscopy of unreacted tyramine polymer and crosslinked tyramine gel (DS 0.92).

Entrapped Enzyme

Gels with entrapped active enzyme could provide a useful delivery vehicle for catalyzing aqueous reactions. Several studies were undertaken in order to determine if HRP remains entrapped and active inside the gel after gel synthesis and swelling. One qualitative method that was used was through the addition of phenol and hydrogen peroxide. After the gel, 300 μL in volume, had swelled and equilibrated in buffer for several days, a drop of phenol was added to the gel and solution (2–3 mLs of buffer at pH 7). The solution was gently mixed for several minutes, then a drop of 0.3% hydrogen peroxide was added. If the enzyme were active either inside or outside the gel, then the gel and/or solution would turn a dark black/brown color due to the polymerization of the phenol. For six tyramine gels, only one showed a color change. The color appeared in the center of a gel that had been synthesized with a high concentration of HRP enzyme (4 mg/mL). In all other cases, no color was seen indicating the absence of active enzyme. This result is in agreement with many cases of aromatic polymerization using peroxidases, where after polymer synthesis the enzyme has been found to be inactive, most likely through peroxide poisoning.[12,18,34] The question of whether the enzyme remained trapped within the gel was qualitatively answered through detection of a peak at 405 nm in UV absorbance measurements. A peak at 405 nm is due to absorbance by the heme group in the peroxidase enzyme. When absorbance measurements were made for the sol fraction studies, the absorbance spectrum from 200–700 nm consistently showed a peak at 405 nm at all concentrations of enzyme (i.e., HRP concentrations used in synthesizing the gels) except those below 1 mg/mL, where the enzyme concentration was probably too low to detect. At concentrations at or above

1 mg/mL, the peak at 405 nm would increase with increasing enzyme concentration, indicating that more enzyme was being extracted from the gel as enzyme concentration within the gel increased. These results are evidence that the enzyme does not remain entrapped within the swelled gel.

CONCLUSION

Poly(aspartic acid) polymers functionalized with aromatic groups were crosslinked in aqueous solution with the use of peroxidase and hydrogen peroxide. Using these mild reaction conditions biodegradable protein gels were formed. In these studies, it was found that gelation and gel properties were dependent on a number of factors, the most notable being type of aromatic functional group, degree of substitution of the aromatic, polymer molecular weight, polymer concentration, and type of enzyme. Through varying these factors, gel properties such as swelling and mechanical strength could be controlled. Having this control is extremely important in the synthesis of "smart" gels where hydrogel properties can be highly varied for a broad range of applications. Biodegradable hydrogels have potential for use in water purification, sensor technology, enzyme immobilization and medical technology such as in drug delivery, wound healing, and tissue engineering such as artificial muscles. The synthesis of biodegradable 'smart' hydrogels under mild enzymatic conditions, while avoiding the use of organic solvents and heavy metals, may provide new options for these materials in these and other applications.

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