

Electron paramagnetic resonance spectroscopy as a novel approach to measure macromolecule–surface interactions and activities of extracellular enzymes

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Received 15 June 2005; received in revised form 31 March 2006; accepted 3 April 2006

Available online 22 May 2006

Abstract

The dynamics of high molecular weight organic matter in marine systems are influenced by molecular conformation, interactions with surfaces and susceptibility to enzymatic hydrolysis, parameters that are difficult to observe experimentally. Here we use electron paramagnetic resonance spectroscopy (EPR) and spin-labeled (SL-) polysaccharides to monitor the sorption of SL-polysaccharides to natural sediment surfaces and to montmorillonite and to observe decreases in polysaccharide size due to enzymatic hydrolysis. SL-pullulan, SL-xylan and SL-maltoheptaose all sorbed rapidly to muddy sediments but not to sandy sediments. SL-pullulan and SL-maltoheptaose also both sorbed to montmorillonite; however, SL-pullulan reached substantially greater final surface loadings than did SL-maltoheptaose. Using EPR has the advantages of being rapid (spectra can be acquired in 100 seconds), non-destructive and functional in complex media, including sediment slurries, muddy water or other optically opaque samples, permitting investigation of the interactions between biomacromolecules, extracellular enzymes and mineral surfaces in aquatic environments.

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Keywords: Electron paramagnetic resonance spectroscopy; Dissolved organic carbon; Enzyme; Hydrolases; Polysaccharides; Sorption; Montmorillonite

1. Introduction

Remineralization processes in the water column affect the spectrum of substrates available to sedimentary heterotrophic microbes, as well as the quantity and nature of carbon that is ultimately buried in sediments (Hedges, 1992). Phytoplankton produce a range of high

molecular weight (HMW) organic compounds, including polysaccharides, proteins and lipid complexes, so degradation of HMW organic matter (OM) is an important first step in the remineralization of marine primary production. However, factors controlling the rates, locations and mechanisms of degradation of HMW OM in aquatic environments are not well understood.

In this study, we demonstrate that electron paramagnetic resonance spectroscopy (EPR) can be used to examine two factors that affect the dynamics of HMW OM: activity of microbial extracellular enzymes, and interactions between organic macromolecules and surfaces.

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Bacteria rely on extracellular enzymes to hydrolyze HMW OM prior to uptake, because the membrane proteins responsible for transporting organic molecules into the cell generally cannot accept molecules larger than trisaccharides or tetrapeptides (Benz and Bauer, 1988). The activity of extracellular enzymes is therefore a potential control on the rate at which HMW OM is remineralized.

Current techniques to measure extracellular enzyme activities in aquatic environments suffer from several limitations. The most frequently used method for measuring extracellular enzyme activities relies on small substrate proxies covalently bonded to a fluorophore, for example methylumbelliferyl glucoside (Somville and Billen, 1983; Mudryk and Skórczewski, 2004). Fluorescence is quenched until the bond between the fluorophore and the substrate proxy is cleaved, so enzyme activity is measured as the rate of increase in fluorescence. However, the three-dimensional structure of a polysaccharide can have a significant effect on the rate at which it is hydrolyzed (Hjerde et al., 1996; Warren, 1996). Since low-molecular weight substrate proxies do not represent the structural complexity of organic macromolecules, enzyme activities measured by this method may not accurately reflect the rates of hydrolysis of HMW substrates. Furthermore, this method is not able to distinguish between activities of enzymes specific to different linkage positions, nor can it distinguish between extracellular and periplasmic enzyme activity (Martinez and Azam, 1996).

Another general approach for measuring extracellular enzyme activities relies on the use of chromatography to determine the size distribution of fluorophore-labeled organic macromolecules after enzymatic hydrolysis (Arnosti, 1996, 2003; Pantoja et al., 1997). Unlike the small-substrate proxy method, this method measures only extracellular, not periplasmic, hydrolysis and measures the rate of hydrolysis of specific macromolecules, rather than substrate proxies. However, sample throughput is relatively slow because a single chromatogram can require 90 min to acquire. Fluorescence polarization measurements of fluorescently labeled polysaccharides can be employed to rapidly screen for the presence of hydrolytic activity (Arnosti et al., 2000), but this method requires substantially higher concentrations of polysaccharide than the chromatographic measurement and cannot be used readily in opaque media.

Associations between HMW OM and mineral surfaces are also potential controls on the rate of HMW OM remineralization, particularly in sediments. The frequently observed correlation between organic matter content and sediment grain size (Tanoue and Handa,

1979) or specific surface area (Keil et al., 1994; Mayer, 1994b) implies that mineral surfaces protect organic matter from remineralization in some manner, but the mechanism for that protective effect has been controversial (Henrichs, 1995; Ransom et al., 1997; Mayer et al., 2004). Novel analytical techniques may permit new experiments to elucidate the relationship between extracellular enzymes, sedimentary organic matter and mineral surfaces, and thereby help explain how some apparently labile organic biochemicals evade remineralization in sediments over long time periods (Cowie et al., 1995).

Electron paramagnetic resonance spectroscopy (EPR) offers an alternative means of measuring enzyme activities and macromolecular interactions with surfaces. EPR measures the absorption of microwave energy associated with a change of spin state of an unpaired electron in the presence of a magnetic field. Molecules containing an unpaired electron (spin labels, most commonly stable nitroxides; Fig. 1) can be covalently bonded to organic macromolecules including lipids, proteins, carbohydrates and nucleotides (Gaffney, 1976). Because the line shape of the EPR spectrum is affected by the chemical and physical environment of the spin label as well as the behavior in solution of the molecule to which it is bonded, EPR can be used to investigate the dynamics of macromolecules (Berliner, 1976).

The breadth of an EPR resonance of a spin-labeled molecule depends on the rate of rotation of the molecule in solution: large, slowly tumbling molecules exhibit broader lines than small, rapidly tumbling molecules due to incomplete averaging of the principal components of the g and hyperfine tensors (Freed, 1976). The rotational correlation time (τ_c), a measure of the characteristic rate of tumbling of a molecule, can be calculated from the linewidth of the EPR resonance (Nordio, 1976). Decreases in the τ_c of a spin-labeled macromolecule as it is cleaved by an enzyme will lead to a decrease in linewidth of the resulting EPR spectrum, so EPR spectra can be

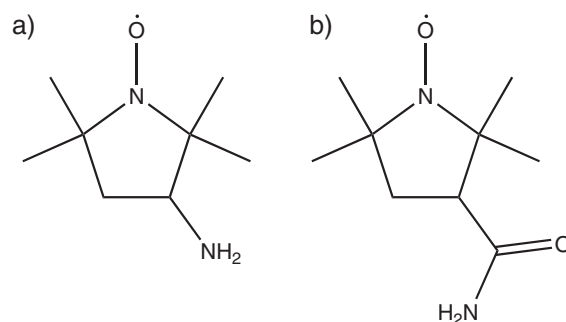


Fig. 1. Structures of (a) 3-amino proxyl and (b) 3-carbamyl proxyl.

employed to detect hydrolysis as well as to estimate the hydrolysis rate. Since EPR employs microwave radiation, it can be applied to optically opaque as well as transparent media and spectra can be acquired rapidly (on the order of 100 s, 1–2 orders of magnitude faster than is possible using chromatographic methods) using environmentally relevant concentrations of macromolecules.

EPR can also be applied using two distinct approaches to measure the extent of surface association of organic molecules. First, association between a macromolecule and a surface decreases the mobility of the macromolecule. This decrease in mobility can appear as an increase in line width and thus rotational correlation time of a spin-labeled macromolecule. In cases where the immobilization is substantial, as when a spin label is sorbed directly to a mineral surface, the signal can become so broad that signal intensity appears to be lost. Second, EPR resonance intensity is sensitive to the concentration of spin label in a sample, so sorption can be measured as the difference between the quantity of spin-labeled macromolecule initially added to a slurry sample and the concentration of that molecule in solution after a given time interval.

Because carbohydrates constitute a large fraction of seawater and porewater DOC (Benner et al., 1992; Arnosti and Holmer, 1999), and because key aspects of the cycling of carbohydrates are not well understood, the experiments described here focused on the dynamics of polysaccharides. We demonstrate that EPR can be used to follow the enzymatic hydrolysis of polysaccharides in solution. Furthermore, we show that EPR can provide information on two specific aspects of carbohydrate dynamics: the effect of polysaccharide chain length on sorption equilibrium and the extent to which the nature of sediment influences polysaccharide sorption.

2. Methods

2.1. Materials and synthesis

Pullulan ($\alpha(1,6)$ -linked maltotriose [$\alpha(1,4)$ linked glucose]; ~ 70 kDa), xylan ($\beta(1,4)$ -linked xylose; 8 kDa) and maltoheptaose ($\alpha(1,4)$ -linked glucose; 1.3 kDa) were obtained from Sigma. Spin labeling was carried out in an analogous fashion to the procedure of Glabe et al. (1983) for fluorescent labeling of polysaccharides, as modified by Arnosti (1995, 1996). In brief, polysaccharides were activated with CNBr at pH > 9.5 for 6 min. Activated polysaccharides were stabilized and separated from unreacted CNBr via gel permeation chromatography (GPC) (18×1 cm column, Sephadex G-25) using 0.2 M $\text{Na}_2\text{B}_4\text{O}_7$ buffer (pH 8.0) as a mobile phase

(flow rate ~ 1.5 ml min^{-1}), and collected in a vial containing 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy ('3-aminoproxyl', Acros Organics) (Fig. 1). After ~ 18 h of incubation at room temperature, labeled pullulan was separated from unreacted spin label by GPC (26×1 cm column, Sephadex G-50) using a phosphate buffer (pH 8.0, 100 mM NaCl + 50 mM phosphate) as a mobile phase. Labeled xylan was separated in the same fashion, using a Sephadex G-25 column. Since labeled maltoheptaose could not be cleanly separated from unreacted proxyl using a chromatographic column, the labeled maltoheptaose was separated from unreacted spin label by extensive dialysis in a Spectra-Por CE 500 Da membrane against milli-Q H_2O . Dialyzed polysaccharides were lyophilized and redissolved in milli-Q- H_2O . Carbohydrate concentration was determined from the mass of lyophilized polysaccharide, which had been dialyzed against milli-Q- H_2O to remove buffer salts. Labeling density was determined by spectrophotometric comparison with standard solutions of free 3-aminoproxyl. Labeling density of spin labeled carbohydrates, expressed as the fraction of monomers bonded to a spin label, was in all cases below the detection limit of 0.6–1.5%.

2.2. Polysaccharide hydrolysis reactions

Enzymatic hydrolysis of pullulan was carried out by reacting 50 μl of a 1.32 g/l^{-1} solution of dialyzed, lyophilized SL-pullulan in phosphate buffer (pH 5.0) with 50 μl of a 0.25 g/l^{-1} solution of pullulanase enzyme (Sigma-Aldrich, from *Klebsiella pneumoniae*) in the same buffer. The resulting solution was drawn into 50 μl capillaries (Corning), which were then sealed with CRITOSEAL[®] and placed within standard 3 mm i.d. quartz EPR tubes. The tubes were then placed and incubated in the cavity of the EPR spectrometer at room temperature. Successive EPR spectra were obtained over the course of the incubation. Instrumental parameters are provided below and in the figure captions.

2.3. EPR measurements

EPR measurements were obtained using a Bruker ER-200D-SRC spectrometer, set to a nominal operating frequency of 9.79 GHz at a power of 10 mW. All measurements were made at room temperature, with a center field of 3481 G, using a sweep width of 60–80 G, sweep times of 50 s–200 s and receiver gain ranging from 4×10^3 to 2.5×10^5 . Time constants ranged from 10 ms to 80 ms and, in all cases, the modulation amplitude was 1 G.

EPR linewidth depends on the rotational correlation time, τ_c , of the spin-labeled molecule, such that larger

Table 1
Definition of symbols

Symbol	Unit	Description	Value	Reference
$\Delta H(0)$	G	Magnetic field separation between the maximum and minimum of the central ($m=0$) line	Variable	n/a
$h(m)$	None	Difference between the maximum and minimum y -value of the m th EPR line (low-field line corresponds to $m=-1$, the central line to $m=0$ and high-field line to $m=1$)	Variable	n/a
H_0	G	Magnetic field at the zero-crossing point of the central line	Variable	n/a
A_{rr}	Hz	rr element of the hyperfine coupling tensor	$9.2639 * 10^7$	Forrester (1989)
a	Hz	Isotropic hyperfine coupling constant	$4.0424 * 10^7$	Forrester (1989)
g_{nn}	n/a	Principle elements of g tensor	2.00222 (g_{pp}), 2.00893 (g_{qq}), 2.00607 (g_{rr})	Forrester (1989)
g_e	None	g factor of the free electron	2.00232	Weil et al. (1994)
β_e	erg·G ⁻¹	Bohr magneton	$9.27 * 10^{-21}$	Atkins (1998)
h	erg·s	Planck's constant	$6.63 * 10^{-27}$	Atkins (1998)
k	J·K ⁻¹	Boltzmann constant	$1.38 * 10^{-23}$	Atkins (1998)
T	K	Temperature	293.15	n/a
η	kg·m ⁻¹ ·s ⁻¹	Viscosity of water	$1.00 * 10^{-3}$	Lide (2004)

and more slowly rotating molecules exhibit broader lines. Rotational correlation time is thus a measure of the mean time for which the spin label assumes any particular orientation in space and can be calculated from two different formulae (Nordio, 1976):

$$\tau_{c(B)} = \frac{15\Delta H(0)g_e \left[\left(\frac{h(0)}{h(1)} \right)^{\frac{1}{2}} - 1 \right]}{16\pi^2 H_0 (A_{rr} - a) \left[g_{rr} - \frac{1}{2} (g_{pp} - g_{qq}) \right]} \quad (1)$$

$$\tau_{c(C)} = \frac{2\Delta H_0 g_e \beta_e \left[\left(\frac{h(0)}{h(1)} \right)^{\frac{1}{2}} + \left(\frac{h(0)}{h(-1)} \right)^{\frac{1}{2}} - 2 \right]}{\pi^2 h (A_{rr} - a)^2} \quad (2)$$

with the parameters defined in Table 1 and spectral parameters illustrated in Fig. 2. (Note that EPR spectrometers report the first derivative of absorption with respect to applied magnetic field.) τ_c was calculated as the geometric mean of $\tau_{c(B)}$ and $\tau_{c(C)}$ (Ottaviani et al.,

1995) and, using the Debye diffusion model, was used to estimate the radius, R , of the molecule, assuming a spherical shape (Nordio, 1976):

$$R = \left(\frac{3\tau_c kT}{4\pi\eta} \right)^{\frac{1}{3}} \quad (3)$$

with parameters from in Table 1.

Nitroxide (spin) concentration is proportional to the area under the EPR absorption signal, which can be obtained by double integration of the EPR line. Spectra were digitized using Un-Scan-It 5.1.6 software (Silk Scientific).

2.4. Behavior of SL-polysaccharides in natural sediments

Seawater and muddy sediments were collected by divers at Cape Lookout Bight, NC, whereas sandy sediments were collected by grab corer from Delaware Bay. 3.0 ml seawater was added to 3.6 g muddy

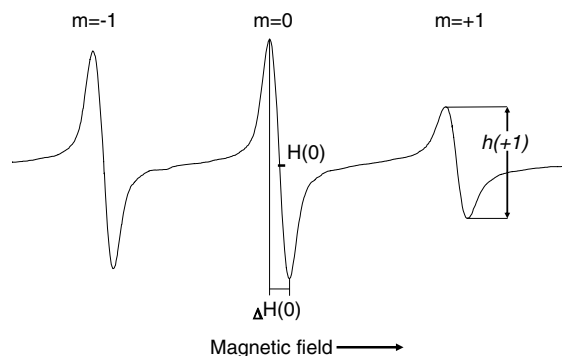


Fig. 2. Parameters of an EPR spectrum necessary to measure rotational correlation time and radius of a spin-labeled molecule.

sediments, and sandy sediments were mixed in the ratio 6.15 g sand:3.0 ml seawater to make master slurries. Subsamples of the master slurries were mixed with SL-pullulan, -xylan or -maltoheptaose in Eppendorf mini-centrifuge tubes, and mixed on a vortex mixer. Seawater and SL-polysaccharides were mixed in the Eppendorf tubes and vortexed. A sample of the solution (the supernatant, for the slurries) was drawn into 50 μl capillary tubes (Corning), sealed with CRITOSEAL[®] and spectra were obtained as described in the previous section. Multiple EPR spectra were obtained from the same capillary tube over the course of 30 min to examine short-term behavior of SL-polysaccharides in seawater and sediments. Seawater+SL-polysaccharide samples were re-analyzed (same capillary tube) after incubation at room temperature overnight. To determine whether hydrolysis occurred over longer timescales in the supernatant of the sandy and muddy slurries, the slurries were incubated overnight at room temperature and then fresh samples were drawn from the supernatant of the Eppendorf tubes into new capillary tubes.

2.5. Sorption of SL-polysaccharides to montmorillonite

Sorption of SL-polysaccharides to montmorillonite was initiated by mixing between 5 and 50 mg montmorillonite K-10 (Aldrich) with 0.85 mg SL-pullulan or 0.21 mg SL-maltoheptaose dissolved in 1.00 ml artificial seawater. The reaction vessels were placed on a wave table to suspend the montmorillonite and thoroughly mix samples. The samples were then centrifuged for 6 min prior to removing $\sim 50 \mu\text{l}$ supernatant for EPR analysis. Mixing was only assumed to occur when the reaction vials were on the wave table, so the time between centrifugation and replacement of the reaction vessels on the wavetable was not counted as part of the total reaction time.

Reversibility of sorption was measured by adding 14.7 mg ml^{-1} unlabeled pullulan (more than is readily soluble) to the supernatant of a mixture containing 50 mg montmorillonite with sorbed SL-pullulan. The mixture was incubated overnight and an EPR spectrum of the supernatant was obtained.

3. Results and discussion

3.1. Solution behavior of SL-polysaccharides

Two polysaccharides, pullulan and xylan, and one oligosaccharide, maltoheptaose, were spin-labeled, and EPR spectroscopy was used to investigate their behavior in solution in comparison to the behavior of the free spin

label. As expected, the EPR line width increased with increasing molecular weight. Free nitroxide tag (3-carbamyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, '3-carbamyl-proxyl', molecular weight=185, Fig. 1) showed the narrowest linewidth, followed by SL-maltoheptaose (MW=1292), SL-xylan (MW \sim 8000) and SL-pullulan (MW \sim 70,000) (Fig. 3). Rotational correlation times and molecular radii calculated employing Eqs. (1)–(3) increased with increasing molecular weight (Table 2). Standard deviation of replicate τ_c measurements were less than 3% of the mean τ_c .

The extent to which the radii of polysaccharides calculated from τ_c are reasonable can be evaluated by calculating the radius from an assumed density (molar mass/partial molar volume) of dissolved polysaccharides. For a wide range of monosaccharides and oligosaccharides, this density is consistently close to 1.54 g ml^{-1} (Adachi and Matsuno, 1997). Assuming that polysaccharides have the same density, the radius of the polysaccharides studied here would be considerably larger: 2.7×10^{-9} m for pullulan (versus 1.0×10^{-9} m from τ_c) and 1.4×10^{-9} m for xylan (versus 4.3×10^{-10} m from τ_c). Several factors could cause τ_c to underestimate the characteristic radius of the polysaccharide. First, these polysaccharides may not assume a sphere-like conformation as assumed by the Debye diffusion model. Second, localized motion of the spin label (i.e., non-rigid body motion) would act to decrease τ_c . The most likely source of such motion is rotation of the spin label relative to the polysaccharide about the bond between the spin label and the polysaccharide. Another potential source is "wiggle" of subsections of the polysaccharide chain, reflecting the fact that the polysaccharide is not rigid. Despite these

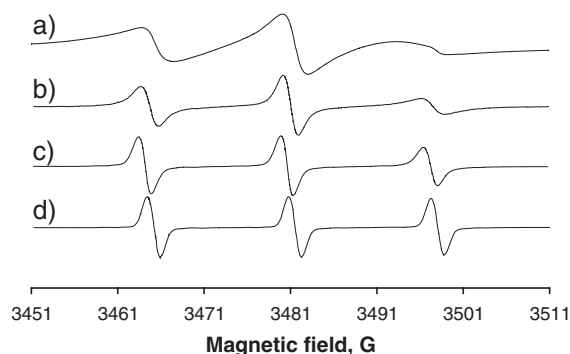


Fig. 3. (a) EPR spectra of SL-pullulan, (b) SL-xylan, (c) SL-maltoheptaose and (d) free spin label (3-carbamylproxyl). EPR parameters for all spectra: scan range=60 G, scan time=100 s, time constant=80 ms; except that the SL-pullulan spectrum had scan time=200 s.

Table 2
Rotational correlation times and radii of spin-labeled molecules of varying sizes

	SL-pullulan	SL-xylan	SL-maltoheptaose	3-CP
Polysaccharide mass (Da)	~70,000	~8,000	1,292	185
Rotational correlation time, $s * 10^{-10}$	8.65	3.77	0.709	0.125
Calculated radius, $m * 10^{-10}$	9.41	7.13	4.09	2.29

deviations from the rigid sphere model, the natural log of the formula weight of the four molecules tested correlated very well to the calculated radius ($r^2=0.9922$, Table 2). Although spin-labeled polysaccharides do not resemble rigid spheres sufficiently to allow accurate calculations of polysaccharide radius from τ_c ; nevertheless, Table 2 shows that the radii calculated using Eq. (2) can be used as a relative measure of molecular weight of SL-polysaccharides.

In the presence of pullulanase, the line width of SL-pullulan became progressively narrower with time (Fig. 4a), reflecting the formation of lower molecular weight fragments having smaller τ_c and radii (Fig. 4b). A comparison of the τ_c of pullulan after 32 min of hydrolysis with those of the spin-labeled polysaccharides and 3-CP (Table 2) indicates that the size of the average pullulan fragment was between 1 and 8 kDa, thus permitting an approximation of pullulanase activity in the sample. This demonstrates that EPR spectra are capable of revealing enzymatic hydrolysis of SL-polysaccharides. Through the use of additional spin-labeled size standards, a more precise determination of pullulanase activity might be made.

The spectra observed during hydrolysis represent a superposition of pullulan molecules hydrolyzed to varying degrees. Since pullulanase is an endo-acting enzyme which cleaves polymers mid-chain (Domań-Pytka and Bardowski, 2004), hydrolysis products will include a size range of smaller polymers and oligomers as the enzyme progressively hydrolyzes the initial polysaccharide (Amosti et al., 1994). The monotonic decrease of line width with increasing hydrolysis time (Fig. 4b) suggests a smooth progression from the high molecular weight parent to intermediate molecular weight structures with no large changes in the dispersion of molecular weights, consistent with the typically observed endo activity of environmental pullulanases (Fig. 1 in Amosti et al., 1994).

3.2. Spin-labeled polysaccharides in seawater and natural sediments

Spectra of SL-pullulan, -xylan and -maltoheptaose were acquired in phosphate buffer, seawater and slurries of sandy and muddy sediments in order to investigate potential effects of medium composition on EPR parameters. Spectra of the individual spin-labeled polysaccharides were identical in phosphate buffer, seawater and a slurry of sandy sediments, and further, did not change over the course of a 12-h incubation, indicating that the SL-polysaccharides did not significantly interact with the dissolved components of seawater (for instance, metal cations or dissolved organic matter) or with the sandy sediments (Fig. 5, Table 3).

The spectra of all three SL-polysaccharides did, however, change rapidly in the presence of muddy sediments

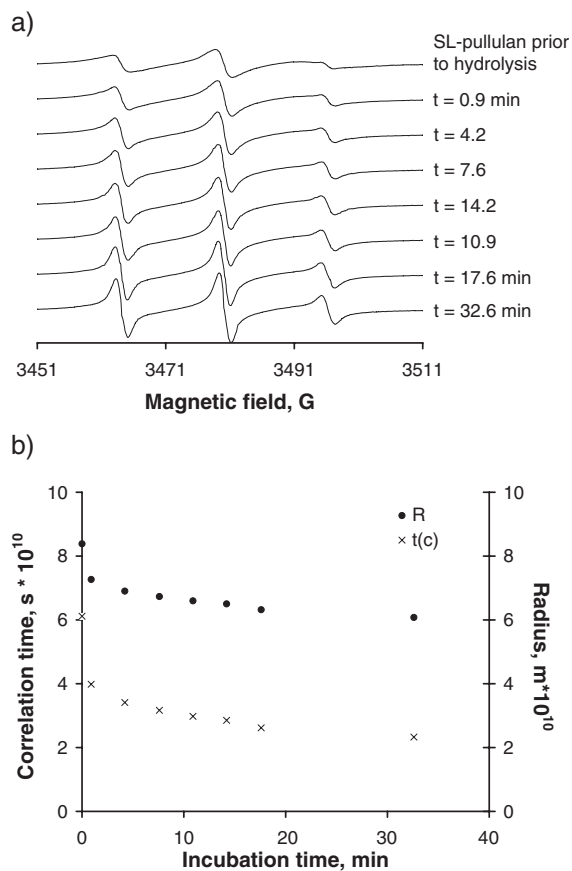


Fig. 4. (a) EPR spectra of SL-pullulan in the presence of pullulanase and (b) corresponding rotational correlation times and radii at various timepoints. $t=0$ sample corresponds to SL-pullulan before the addition of pullulanase. A control sample, containing SL-pullulan but no pullulanase, did not show a change in EPR line shape (not shown). EPR parameters: scan range=80 G, scan time=200 s, time constant=80 ms.

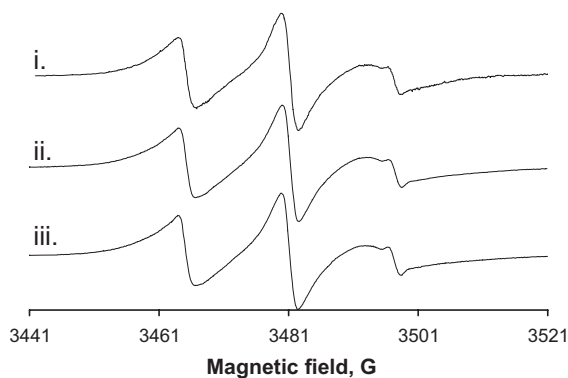


Fig. 5. EPR spectra of SL-pullulan in (i) phosphate buffer, (ii) seawater and (iii) a slurry of sandy sediments. Spectra were obtained as soon as possible after introduction of the sediments. EPR parameters: scan range=80 G (60 G for SL-pullulan in phosphate buffer), scan time=200 s, time constant=80 ms.

(Fig. 6a–c). For each polysaccharide, signal intensity (measured as $h(0)$) decreased substantially within minutes of introduction of muddy sediments (Fig. 7a). Concurrently, rotational correlation times increased by as much as 25% relative to the corresponding SL-polysaccharide dissolved in seawater (Fig. 7b). The rapid decrease in signal intensity and slight change in spectral linewidth suggest that there were significant interactions between SL-polysaccharides and muddy sediments, in contrast to the lack of interaction between SL-polysaccharides and dissolved components of seawater or sandy sediment surfaces. A small part of the decrease in $h(0)$ in the presence of muddy sediments can be explained by line broadening as evidenced by the increased τ_c . However, under the conditions that apply here, the relationship between $h(0)/h(1)$ or $h(0)/h(-1)$ and τ_c is very nearly linear, such that a 10–20% increase in τ_c will produce an equal decrease in $h(0)$, which cannot explain the magnitude of the large observed decreases of $h(0)$. Correlation times of SL-polysaccharides in the presence of muddy sediments increased by 10–25%, whereas $h(0)$ decreased by 35–100%. The fact that the decreases in resonance intensity observed were far larger than the

Table 3

Rotational correlation time for three substrates in various media, seconds

	SL-pullulan	SL-xylan	SL-maltoheptaose
In phosphate buffer	5.82E-10	4.17E-10	1.49E-10
In seawater	6.08E-10	3.68E-10	1.46E-10
In sandy slurry, initial	5.84E-10	3.79E-10	1.40E-10
In sandy slurry, after overnight incubation	6.51E-10	4.03E-10	1.45E-10

increases in correlation time indicates that signal intensity decreased. We attribute this reduction in signal to strong sorption of the polysaccharide to the large sediment particles, thus immobilizing the spin labels and causing the spectrum to broaden to such an extent that signal intensity appears to be lost. The rapid decrease in $h(0)$ (beyond the fraction that can be explained by line broadening) therefore indicates that a substantial fraction of the SL-polysaccharides sorbed to surfaces of the muddy sediments on a timescale of minutes. Sorption of

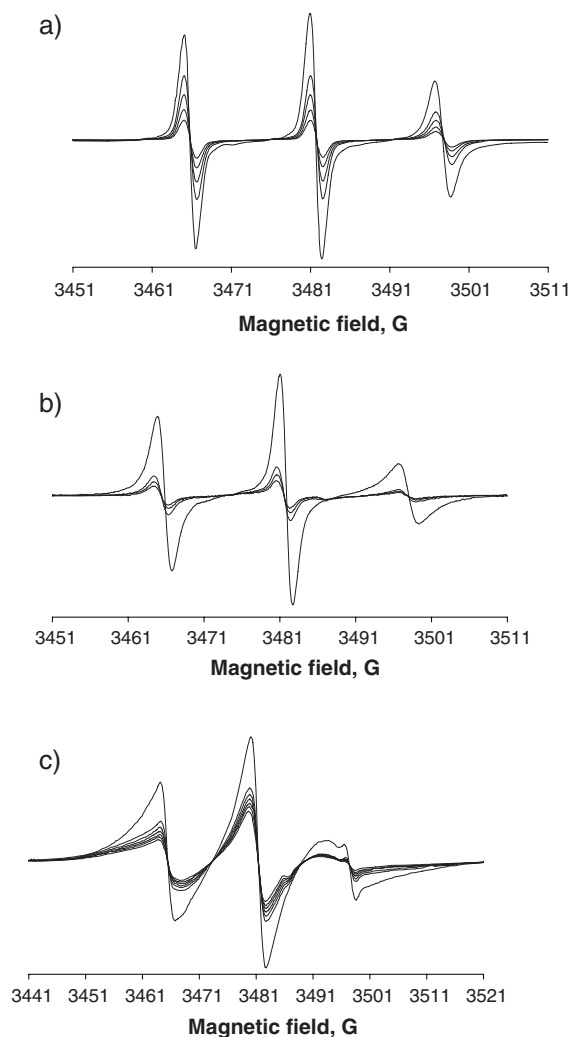


Fig. 6. Successive spectra of (a) SL-maltoheptaose, (b) SL-xylan and (c) SL-pullulan in the presence of a slurry of muddy sediments. In all cases, each successive spectrum is less intense than the previous one. The most intense spectrum is of the SL-polysaccharide in seawater. Spectra were taken (for SL-maltoheptaose) after 10, 13, 16 and 19 min (for SL-xylan) after 5, 8 and 11 min, and (for SL-pullulan) after 1.6, 4.9, 8.3, 11.6, 14.9 and 23.3 min. EPR parameters: scan range=60 G (80 G for SL-pullulan), scan time=200 s, time constant=80 ms.

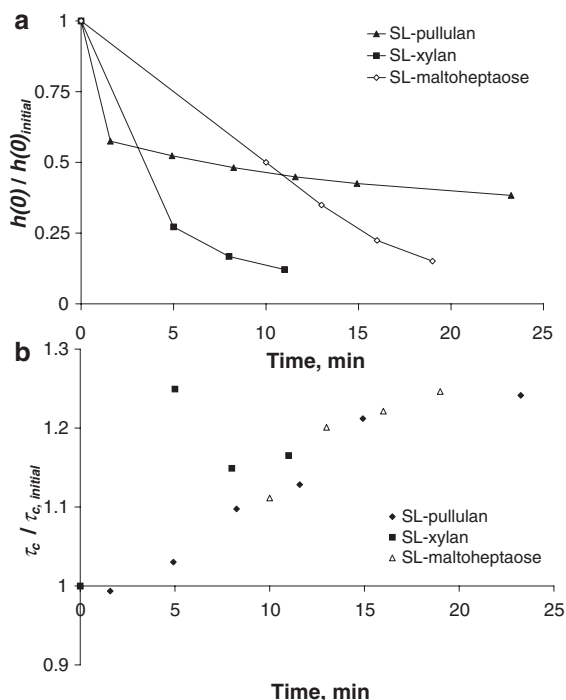


Fig. 7. (a) Relative change in $h(0)$ of SL-polysaccharides in the presence of muddy sediments. $h(0)$ of the polysaccharide in seawater was taken as the reference value $h(0)_{initial}$. (b) Relative changes in rotational correlation times of SL-polysaccharides in the presence of muddy sediments. Rotational correlation times of the polysaccharide in seawater were taken as the reference value.

SL-pullulan and SL-maltoheptaose appeared to be biphasic, with a rapid loss of signal intensity in the first several minutes (before the first measurement was obtained), followed by slower loss of signal intensity (Fig. 7a). Biphasic sorption kinetics suggest that two distinct mechanisms, with two different rate constants, were responsible for sorption.

Reduction of the nitroxide spin probe could be an alternate explanation for the loss of resonance intensity of the SL-polysaccharides in the presence of sediments. In these experiments, however, reduction of the spin probe was extremely unlikely. First, the sediments used were suspended in a thin slurry with oxygenated seawater. The persistence of signal intensity in the sandy slurry demonstrates that nitroxide spin probes are stable in the presence of seawater. It is possible that reducing substances (e.g. reduced metals or glutathione) from the sediments could reduce the nitroxide to hydroxylamine, but in oxygenated solution O_2 rapidly reoxidizes the hydroxylamine to the original nitroxide form (Kocherginsky and Swartz, 1995).

In separate experiments, sediment was added to 10–20 μM solutions of three spin probes (3-aminoproxyl, 3-

carboxyproxyl and 3-carbamyl proxyl) in seawater. EPR resonance intensity decayed at different rates (data not shown). After 18 h, Cu^{2+} (as cupric sulfate) was added to a final concentration of 1 μM . Cu^{2+} catalyzes the rapid reoxidation of nitroxides in the presence of O_2 (Schwartz et al., 1979). After addition of Cu^{2+} , signal intensity did not return. The fact that signal intensity decreased even though the solution was oxygenated, combined with the failure of signal to return in the presence of Cu^{2+} , suggests that reduction was not the cause of the decrease in signal intensity. Oxidation of the nitroxide was unlikely under these conditions, in the absence of a strong oxidant such as H_2O_2 or NaClO (Kocherginsky and Swartz, 1995).

The relatively small (10–25%) initial increase in correlation time is suggestive of weak immobilization of SL-macromolecules (possibly by interactions among SL-polysaccharides (Chin et al., 1998), or by formation of weak associations between SL-polysaccharides and mineral surfaces, prior to the formation of strong associations that totally immobilized the spin labels and caused such great broadening that the signal appeared to be lost.

The observation that SL-pullulan, SL-xylan and SL-maltoheptaose all sorbed to fine-grained, muddy sediments, but not to the coarser sandy sediments is consistent with reports that organic matter loading on continental shelf sediments tends to be proportional to specific surface area (Mayer, 1994a). Alternately, since mineral surface chemistry influences the types of bonds that can form between organic matter and mineral surfaces (Tanoue and Handa, 1979; Satterberg et al., 2003), the surface of the sand grains may have been less amenable than the muddy sediments (including any organic matter already associated with them) to sorption by polysaccharides.

3.3. Interactions between spin-labeled polysaccharides and montmorillonite surfaces

Montmorillonite has frequently been used as a model clay in studies of the interactions of organic molecules with clay minerals (Parfitt and Greenland, 1970; Olness and Clapp, 1975; Bradbury and Baeyens, 2005). The spectra of SL-pullulan and SL-maltoheptaose in the presence of montmorillonite decreased in intensity ($h(0)$) by 25–100% over the course of 24 h (Fig. 8). The decrease in $h(0)$ of SL-pullulan and SL-maltoheptaose in the presence of montmorillonite is interpreted as a change in signal intensity due to sorption. In a separate experiment, Cu^{2+} was added solutions of spin probes in the presence of montmorillonite (as described in Section

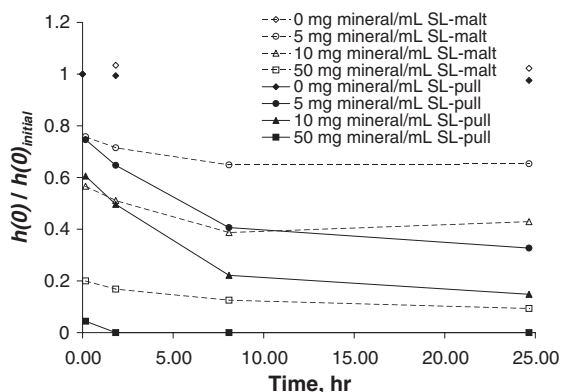


Fig. 8. Relative changes in the $h(t)$ of SL-pullulan and SL-maltoheptaose dissolved in ASW in the presence of montmorillonite, as a function of time. $h(0)$ of the appropriate polysaccharide immediately after it was dissolved in ASW was used as the reference value. In order to facilitate comparison of EPR spectra, the concentration and quantity of SL-polysaccharide solution was kept constant, while the quantity of montmorillonite was varied. Unbroken lines and filled symbols indicate SL-pullulan; dashed lines and open symbols indicate SL-maltoheptaose. Diamonds: zero montmorillonite present, circles: 5 mg montmorillonite/ml polysaccharide solution, triangles: 10 mg ml, squares: 50 mg ml^{-1} .

3.2), in which signal intensity had decayed with time, resonance intensity did not return. Given the lack of reductants in the montmorillonite–water–SL-polysaccharide system, redox changes of the nitroxide probe were ruled out. The ratio $h(t)/h(0)_{\text{initial}}$, where $h(0)_{\text{initial}}$ is the $h(0)$ of the appropriate SL-molecule in seawater, was therefore interpreted as the relative concentration of polysaccharide in solution; the balance is presumed to be sorbed to mineral surfaces.

The kinetics of sorption to montmorillonite were biphasic for both SL-pullulan and SL-maltoheptaose, similar to the observation with muddy sediments. During a rapid initial phase lasting less than 10 min, most of both molecules sorbed to the mineral surface. Sorption continued at a much slower rate afterwards. Sorption of SL-maltoheptaose reached an apparent maximum after 8 h, with very little change in concentration of SL-maltoheptaose in the supernatant between 8 and 24 h. All of the SL-

pullulan in the sample containing the most montmorillonite was removed from the supernatant within 2 h. In the samples containing less mineral surface area, the fraction of sorbed SL-pullulan also appeared to approach a maximum because the rate of sorption decreased with time; however, maximum loading was not reached during the course of the experiment. For a given quantity of mineral surface area, the fraction of SL-pullulan sorbed to the mineral surface was greater than the fraction of SL-maltoheptaose sorbed, with the exception of the two samples with lower SL-pullulan concentrations at the first timepoint.

After overnight incubation, no SL-pullulan was detected in the supernatant of a slurry that initially contained dissolved, unlabeled pullulan and SL-pullulan sorbed to montmorillonite. There was therefore no detectable exchange between sorbed SL-pullulan and dissolved, unlabeled pullulan on the timescale measured.

The fact that no exchange was observed between SL-pullulan sorbed to montmorillonite and unlabeled pullulan in the dissolved phase suggests that sorption for this polysaccharide is not easily reversible on short timescales. The observed kinetics may therefore reflect the irreversible loading of polysaccharides to saturation on mineral surfaces, rather than an approach to equilibrium.

Montmorillonite had greater capacity to sorb SL-pullulan than SL-maltoheptaose after 24 h, both in terms of mass of polysaccharide sorbed per unit surface area, and as a fraction of the total polysaccharide in the system. Longer polymers sorb more irreversibly to minerals, because the probability that all sorbed segments of a polymer will simultaneously detach from the surface of a mineral grain decreases as polymer length increases (Collins et al., 1995). As few as 30% of the monomers in a surface-bound polymer may be directly associated with the surface; the rest form ‘loops’ or ‘trains’ (unsorbed sections of polymer) away from the mineral surface (Theng, 1982). For some neutral polysaccharides sorbed to Na-montmorillonite, the majority of the monomers are in ‘loops’ and ‘trains’ rather than sorbed directly to the surface (Olness and Clapp,

Table 4
Polysaccharide loading on montmorillonite as a function of mineral surface area

	Initial polysaccharide concentration in solution	Quantity of montmorillonite present (mg)	% polysaccharide sorbed after 24 h	Final polysaccharide loading (mg carbon m^{-2})
Maltoheptaose (1.2 kDa)	0.21 mg matter ml^{-1}	5.45	34.7%	0.02
	solution 0.093 mg C ml^{-1}	10.60	57.1%	0.018
		49.14	90.7%	0.0064
Pullulan (70 kDa)	0.85 mg matter ml^{-1} solution 0.38 mg C ml^{-1}	5.26	67.3%	0.176
		9.73	85.2%	0.12
		52.0	100%	0.026

1975). Although it is not clear how restricted in space spin-labels attached to ‘loops’ or ‘trains’ would be, a smaller fraction of directly surface-associated monomers in SL-pullulan than SL-maltoheptaose could cause montmorillonite to bind more monomers of pullulan than maltoheptaose per unit surface area.

Surface loadings of pullulan and maltoheptaose on montmorillonite were substantially lower than organic carbon surface loadings reported for continental shelf sediments (Table 4). Shelf sediments typically show organic carbon surface loadings of 0.86 mg C m^{-2} mineral surface area (Keil et al., 1994; Mayer, 1994b), while the largest loadings of SL-polysaccharides on montmorillonite were 0.18 mg C m^{-2} for pullulan and $0.053 \text{ mg C m}^{-2}$ for maltoheptaose. In experiments in which natural organic matter was sorbed to clean montmorillonite, Amarson and Keil (2000) also reported sparse equilibrium organic carbon loadings. Clean montmorillonite in the presence of DOC concentrations up to $\sim 25 \text{ mg C l}^{-1}$ came to an equilibrium loading of only $0.0015 \text{ mg C m}^{-2}$, or $<0.5\%$ of typical continental margin sediment organic matter loadings. Montmorillonite may have less capacity to sorb organic matter than typical bulk marine sediments. One reason for this effect could be montmorillonite’s low point of zero charge (pH 2.5) relative to calcite and oxides of iron and aluminum (pH 7–10) (Stumm and Morgan, 1996).

4. Conclusions

EPR was used to monitor dynamics of two spin-labeled polysaccharides and one spin-labeled oligosaccharide in solution. Changes in correlation time of SL-pullulan due to enzymatic hydrolysis allowed observation of enzymatic hydrolysis in quasi-real time. Changes in spectral intensity allowed observations of kinetics of sorption of SL-polysaccharides to mineral and sediment surfaces.

All three SL-oligo and -polysaccharides were observed to associate with muddy sediments, but not with sandy sediments. These differences are likely due to a combination of the effects of differing mineralogy, specific surface area and quantity, and possibly nature of organic matter previously associated with the mineral grains. Biphasic kinetics of sorption to montmorillonite were observed, with a rapid initial phase lasting less than 10 min followed by a slower phase lasting longer than 6 h, suggesting that two distinct mechanisms of sorption were important.

EPR combines unique advantages over other techniques for measuring the dynamics of organic macromolecules: it is rapid, non-destructive and measurements can be made with specific macromolecular substrates.

EPR spectra of spin-labeled polysaccharides yield similar information about the size of polymers as previously established chromatographic methods. However, EPR data can be acquired much more rapidly than data using those chromatographic methods, since EPR spectra can be acquired in 1–4 min. Enzymatic hydrolysis reactions can be observed in ‘real time’ by EPR, while chromatographic analysis requires further sample preparation. EPR can readily function in complex media including mixed-phase environments such as the nepheloid layer, particle-rich estuarine water and marine aggregates. Furthermore, EPR’s ability to measure both enzymatic hydrolysis of biomacromolecules and their interactions with surfaces could allow new experimental approaches to untangle the complex relationships between DOC, extracellular enzymes and minerals.

Acknowledgements

This work was supported by funding from NSF (OCE-0323975) and the Petroleum Research Fund (to CA), and the Office of Naval Research (N00014-02-10654, NVB). We thank Valerie Whalen and Tamas Kalai for assistance with the initial EPR measurements. Min Jia provided technical assistance with the EPR. ADS is grateful to Bela and Asgar Ali for food, lodging and much hospitality during his work at the University of Maryland.

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