

Direct visualization of clay microfabric signatures driving organic matter preservation in fine-grained sediment

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Abstract

We employed direct visualization of organic matter (OM) sequestered by microfabric signatures in organo-clay systems to study mechanisms of OM protection. We studied polysaccharides, an abundant class of OM in marine sediments, associated with the nano- and microfabric of clay sediment using a novel application of transmission electron microscopy, histochemical staining (periodic acid-thiosemicarbazide-silver proteinate), and enzymatic digestion techniques. We used two experimental organo-clay sediment environments. First, laboratory-consolidated sediment with 10% chitin (w/w) added was probed for chitin before and after digestion with chitinase. Second, fecal pellets from the polychaete *Heteromastus filiformis* were used as a natural environment rich in clay and polysaccharides. Sections of this material were probed with silver proteinate for polysaccharides before and after digestion with a mixture of enzymes (amylase, cellulase, chitinase, dextranase, and pectinase). In both environments, chitin or other polysaccharides were found within pores, bridging clay domains, and attached to clay surfaces in undigested samples. Digested samples showed chitin or polysaccharides more closely associated with clay surfaces and in small pores. Our results imply protective roles for both sorption to clay surfaces and encapsulation within clay microfabric signatures. © 2007 Elsevier Ltd. All rights reserved.

1. INTRODUCTION

The flow of organic matter (OM) has been studied both in marine sediments and in terrestrial soils, but it is less well understood in marine sediments (Hedges and Oades, 1997). Organic matter entering marine sediments and terrestrial soils is variously degraded, but in both environments it exists in a refractory state closely associated with minerals (Hedges and Oades, 1997; Baldock and Skjemstad, 2000; Baldock et al., 2004; Hebbing et al., 2006). The bulk of this refractory OM is found in those portions of sediments and soils with the highest surface areas, i.e., with the clay

minerals (Kahle et al., 2002; Hebbing et al., 2006; Kennedy et al., 2006).

At least two types of interactions have been proposed to explain OM protection via association with minerals: Hypothesis 1, direct adsorption onto clay surfaces, and Hypothesis 2, encapsulation in clay microfabric signatures (reviewed in Sollins et al., 1996; Baldock and Skjemstad, 2000; Six et al., 2002). Hypotheses for other types of protection, e.g., humification and exclusion of oxygen (Sollins et al., 1996; Baldock and Skjemstad, 2000; Six et al., 2002), may also be to some degree related to OM protection by clay. Many of the proposed, specific mechanisms are complementary to one another rather than competitive (Sollins et al., 1996; Baldock and Skjemstad, 2000; Six et al., 2002; Baldock et al., 2004; Mayer, 2004; Hebbing et al., 2006). Many studies addressing adsorption to mineral surfaces have considered the importance of the mineral

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composition of clay by discussing divalent cations, negatively charged clay faces, and the various organic moieties with which those charged particles interact (Sollins et al., 1996). Others have studied the interaction of enzymes with OM attached to clay surfaces, enzymes becoming attached to clay, and the catalytic action of clay surfaces on OM (McLaren and Estermann, 1956; Sørensen, 1972; Romanowski et al., 1991; Naidja et al., 2000). The collective results of these studies have been equivocal (or at least eclectically derived) with respect to understanding the way in which adsorption to a clay surface can protect OM.

Mayer (1994a,b) discovered a correlation between the surface area of many sediments and soils and the quantity of refractory OM which is slightly less than 1 mg-organic carbon/m² of surface. He referred to this phenomenon as ‘monolayer equivalent’, but stressed the unlikelihood that an actual monolayer would exist. Indeed, Ransom et al. (1997) showed with transmission electron microscopy (TEM) that OM on the surfaces of sediments fitting the monolayer equivalent relationship was in discontinuous lumps, not a monolayer; and much of the sediment surface area was devoid of any OM. Ransom et al. (1998a) found that the abundance of OM was associated with clay minerals such as smectite with high surface activity and surface area. Mayer (1999, 2004) then studied the discontinuous distribution of OM on sediment surfaces. Noting that most of the surface area of many sediments and soils is associated with pores less than 8 nm across, he tested whether OM would largely be protected in these very small spaces. He demonstrated that very little OM was actually in pores less than 8 nm across, leading to the hypothesis that,

“the network of pores that allow access to organic matter within the aggregates may include ‘throats’ of small mesopore [2–50 nm] size. These ‘throats’ would be formed by contacts among crystallites or domains. In this scenario, the mesopore exclusion hypothesis may still apply”.

Few studies of the protection of OM have included direct observation to help elucidate the various active mechanisms of protection. Most studies have instead relied on bulk analysis. Early work by Foster (1981, 1985) on soil used a specific probe to visualize polysaccharides with TEM. Many authors have argued for the importance of direct observational approaches (Hedges and Oades, 1997; Kaiser and Guggenberger, 2000).

Clay minerals likely play different roles at different scales of time and space from initial scavenging of OM to inhibition of diffusion. Our perception of these roles likely is influenced by the scale of observation with which we test these mechanisms. In the present paper we focus on the mechanisms related to nanoscale (100s of nm) physical associations between OM and clay mineral grains. Nano- and microfabric are best assessed via direct observation at nanometer scale by TEM. Over the last several decades, progress has been made in the visualization and understanding of fine-grained marine sediment microfibrils using TEM and scanning electron microscopy (SEM) (Bennett et al., 1977, 1981, 1991, 1999, 2004; Foster, 1981, 1985; Lavoie et al., 1995; Ransom et al., 1997, 1998b; Curry et al., 2005).

Organic material has frequently been found associated with pore spaces between clay domains suggesting that protection is associated with this level of organization. Nano- and microfabric signatures that may influence OM preservation in marine sediments have been observed and identified in TEM and SEM photographs and include: (1) fecal pellet “skin-wall” and intra-pellet aggregates; (2) aggregates with very small intravoids filled with OM and pore water; (3) “onion-skin” microfabric developed around individual prokaryotes, colonies of prokaryotes, and extracellular OM; (4) microfabric of aggregates and linking chains of domains in fine-grained marine deposits; and (5) preferred clay particle orientation developed from consolidation and water loss in fine-grained muds (Pusch, 1970; Bennett et al., 1981, 1989, 1991, 1996, 1999, 2004; Bennett and Hulbert, 1986; Ransom et al., 1997; Bohlke and Bennett, 1980; Lavoie et al., 1996).

Here we expand Foster’s (1981, 1985) approach for visualizing polysaccharides in mineral fabrics by combining his technique with nanoscale imaging and enzymatic digestion for studying OM preservation in marine sediments. We used laboratory-consolidated and natural sediments with controlled enzymatic digestion of polysaccharides. This combination of techniques allowed us to address the manner in which clay microfabric signatures enhance preservation and sequester OM in marine sediments. In support of other reports, but with the novelty of direct visualization, we examine the spatial patterns by which chitin and other polysaccharides are protected against biological recycling in fine-grained sediments by clay microfabric signatures.

Because of the casual use of terms by members of the scientific community on the topic of fine-grained sediment microstructure, we present a brief review of accepted definitions in this research field. The purpose of this review is to enhance reader understanding, clarification of concepts, and results discussed in this paper on fabric, physico-chemistry, processes, and mechanisms that drive OM preservation. Historically, clay fabric was studied and described predominantly at the micrometer scale and the terms used generally referred to clay microfabric. With the advent of high resolution imaging using TEM and improved sample preparation, fabric studies have been extended to nanometer scale thereby enabling scientists to observe and study OM at this smaller scale. Thus, the definitions of clay fabric and physico-chemistry apply to both nano- and microfabric in this study.

Terminology and definitions review

1. Clay microstructure, by definition, refers to the fabric and physico-chemistry of fine-grained sediment. The clay fabric is the orientation and arrangement or spatial distribution of the solid particles and the particle-to-particle relationships. The physico-chemistry relates to the interparticle forces of the sediment that arise from gravitational forces and electrical forces of the particles and the surrounding fluids (Lambe, 1953, 1958; Mitchell, 1956; Foster and De, 1971; Bennett et al., 1977). Electrical forces dominate gravitational forces in surficial sediments including marine sediments. High concentrations of hydrated ions interact electro-kinetically with the clay

particles (Bennett and Hulbert, 1986) and significantly affect forces of interaction among clay particles. In addition, OM has a significant influence on the strength of interparticle bonds in surficial clay sediments (Pusch, 1973; Bennett et al., 1999).

2. A clay domain (Moon, 1972) is defined as a multiplate particle composed of parallel or nearly parallel plates stacked as sheets in a book or with some offset (stair-stepped arrangement). A domain has considerable structural integrity and behaves in a functional sense as a unit particle until the parallel face-to-face arrangement fails under an applied stress such as consolidation and/or shearing (Bennett et al., 1991). High resolution TEM studies have revealed that most sediments are composed of domains, aggregates, and linking chains rather than single plate particles. A “particle” can be defined in terms of its morphology as well as its function (Bennett et al., 1991) which is important when understanding and describing motion of particles under applied stress and convection and diffusion through sediment.
3. Processes and mechanisms. During early sediment diagenesis and the developmental history of clay fabric, the energy sources that result in sediment particle associations, reorientation, and disaggregation are expressed in terms of processes and mechanisms (Bennett et al., 1991). Two or more mechanisms constitute the broader classification termed process. Mechanisms are characterized by the specific energy sources that drive nano- and microfabric development. The fundamental processes in which the mechanisms operate are described as (a) physico-chemical, (b) bio-organic (including both living and non-living), and (c) burial diagenesis (Bennett et al., 1991).
4. A microfabric “signature” is a well-defined, identified arrangement and orientation of solid particles (usually domains) formed by the energy sources associated with specific processes and mechanisms that drive microfabric development (aggregates, clusters, floccules, chains, etc.). Examples are the “onion-skin” fabric of a face-to-face and edge-to-edge arrangement of particles that form around large grains (Collins and McGown, 1974) and highly oriented face-to-face and edge-to-edge domains arranged in a swirl pattern common in remolded sediment (Bennett et al., 1977; Bohlke and Bennett, 1980).
5. We introduce the term nanofabric to refer to portions of the fabric that would be measured in the range of one to hundreds of nanometers. The ability to visualize this level of organization and the fact that mechanisms of OM protection that have been cited in the literature frequently operate at this level make the introduction of this term timely and appropriate. Nano- and microfabric refer to the arrangement, orientation, and association of the solid particles and the differentiation here is to identify the predominant particle sizes under consideration and discussion, because of the rather large size differences of the particles (micro versus nano). The particle sizes have profound effect on the associated physical chemistry, organic chemistry, and geochemistry generally, including the processes and mechanisms driving fabric development and the collective influence on diffusion, convection, OM sequestering, and bulk physical and mechanical properties.

2. MATERIALS AND METHODS

2.1. Laboratory consolidated organo-clay sediment

We created laboratory-consolidated sediment from illite (90% w/w) and smectite (10% w/w; Wards Natural Science Establishment), from which particles $>62.5\ \mu\text{m}$ were removed by sieving. This clay was mixed with chitin (Sigma), a common polysaccharide, to a concentration of 10% (w/w). Chitin concentrations of 1% and 5% were also used, but not reported, since the lower concentrations confounded finding large numbers of sections of material with even distribution of visualized chitin. The clay-organic material was mixed and saturated with seawater (salinity = 23.1) and degassed in vacuum. Physical property measurements were made for two subsamples. Water contents were determined for calculation of porosity (n , ratio of the volume of the voids to the total volume) and void ratio (e , ratio of the volume of voids to the volume of solids) before laboratory consolidation and following each consolidation load (end of each incremental uniaxial load). Samples were consolidated uniaxially and incrementally (load doubled daily) to a final stress of 732 kPa (6.9 kPa = 1 psi; [Electronic Annex EA-1](#)) to compact the mud slurry and to develop significant cohesion of the samples for processing and TEM observation. Standard techniques for laboratory consolidation and the measurement of physical properties of fine-grained sediment can be found in textbooks on soil mechanics. Wet bulk density was also determined for each sample by standard mass–volume measurements ([Electronic Annex EA-1](#)). Illite/smectite samples were consolidated over a course of weeks. Although the consolidation was not done aseptically, no bacteria were observed in our electron micrographs. A subsample of the consolidated sediment was immersed in chitinase ([Table 1](#)) in a 0.25 M MES (2[*N*-morpholino]ethanesulfonic acid) buffered solution, pH 6.0 (to maximize enzymatic activity), for 24 h at 37 °C (approximate optimum temperature for this enzyme); a control sample was left undigested. Samples were dehydrated (interstitial water removed; 50%, 70%, 85%, 95%, and 100% ethyl alcohol followed by several changes of amyl acetate, purged with liquid CO₂ to remove the amyl acetate, and critical point dried), infiltrated with resin ERL 4206 ([Spurr, 1969](#)) under vacuum (Bennett et al., 1977; [Baerwald et al., 1991](#)), and cured for 36 h at 70 °C. This procedure is technically difficult, but is necessary to preserve the structural integrity of the fabric.

2.2. Natural organo-clay fecal pellet

We studied natural sediment in the form of fecal pellets from the marine capitellid polychaete *Heteromastus filiformis*. Fecal pellets were processed in the laboratory on the day the worms were collected from the field (Ocean Springs, Mississippi, USA). Fecal pellets were chosen because we observed that they were rich in polysaccharides and had their outer clay domains oriented to form relatively dense-walled microfabric signatures by the peristaltic action of gut muscles, shear with the gut lining, and/or contraction of the peritrophic membrane. Thus, our objective was to observe the

Table 1
Enzyme parameters including Stokes' radii

Enzyme	Biological source ^a	Conc. (mg/ml)	Activity ^a (U/mg)	Molecular weight (kD)	Calculated Stokes' radii (nm) ^b	Representative Stokes' radii (nm) ^c
Chitinase	<i>Streptomyces griseus</i>	1	200×10^3	30 ^a	2.5	—
Cellulase	<i>Aspergillus niger</i>	1	0.3	26 ^d	2.4	4.0 ^h , 4.4 ⁱ , 6.25 ^j
α -Amylase	<i>Aspergillus oryzae</i>	1	150	51 ^e	3.0	4.1 ^k
Pectinase	<i>Rhizopus</i> sp.	1	400	38.5 ^f	2.7	5.45 ^l , 5.87 ^l
Dextranase	<i>Chaetomium erraticum</i>	0.1	400	70 ^g	3.4	—

^a Based on Sigma catalogue.

^b Method from Uversky (1993).

^c Similar enzymes from different sources for comparison.

^d Hurst et al. (1977).

^e Takagi (1981).

^f Elegado and Fujio (1994).

^g From *Chaetomium gracile*, Hattori et al. (1981).

^h Smriti and Sanwal (1999).

ⁱ Chatterjee et al. (1997).

^j Chatterjee and Sanwal (1999).

^k Wittmann et al. (1998).

^l Fish and Davis (2004).

potential of the microfabric signatures formed in the pellet to preserve polysaccharide from hydrolytic enzymatic attack. Fecal pellets were immersed in a solution containing the five enzymes chitinase, cellulase, α -amylase, pectinase, and dextranase (Table 1) in a 0.25 M sodium acetate or MES buffered solution, pH 6.0, for 24 h at 37 °C (approximate optimum temperature for these enzymes); a control sample was not subjected to digestion. Samples were dehydrated (50%, 70%, 85%, 95%, and 100% ethyl alcohol followed by several changes of acetone), infiltrated with resin ERL 4206 (Spurr, 1969), and cured for 36 h at 70 °C. This procedure, common to processing of biological material, was used because of the anticipated high level of OM in the pellets compared to the laboratory-consolidated samples.

2.3. Visualizing polysaccharides

Histochemical studies of marine sediments indicate that carbohydrates are visually more abundant than proteins, lipids, or other organic compounds (Frankel and Mead, 1973; Whitlatch, 1974; Whitlatch and Johnson, 1974). A periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) procedure was developed for TEM by Thiéry (1967) for use in biological sciences for general carbohydrate staining and applied to soil systems by Foster (1981, 1985).

We used ultrathin sections (ca. 90 nm) of our illite/smectite preparation and of fecal pellets placed on gold-coated nickel grids. These grids were floated in periodic acid (PA) to oxidize 1,2-glycol linkages of the carbohydrates to form free aldehyde groups. The grids were then floated in a solution of thiosemicarbazide (TSC) which attaches to free aldehydes and were then floated in silver proteinate (SP) solution which attaches to TSC. The PA-TSC-SP procedure is specific for (pH) neutral carbohydrates, mucopolysaccharides, and glycoproteins (Hayat, 2000). The silver particles are 3–5 nm in diameter, although in the presence of high concentrations of polysaccharide, aggregates of silver can occur (Hayat, 2000). The fine particulate nature of this stain makes it suitable for visualizing polysaccharides occupying the nano- and micrometer ranges of pore space developed in clay domains that form microfabric signatures. Our results for both laboratory-consolidated sediment and for fecal pellets were based on observations from more than 400 photographs plus hours of TEM observations.

We ran three controls recommended by Hayat (2000): (1) sections were floated in PA to create free aldehyde groups, but TSC was omitted to verify that SP did not bind nonspecifically to aldehydes, (2) PA and TSC were omitted to verify that SP did not bind nonspecifically to anything, and (3) PA treatment was omitted and TSC was included to check the level at which silver would bind without free aldehyde groups created deliberately.

2.4. Quantitative estimation of polysaccharides surviving enzymatic digestion

Quantifying the PA-TSC-SP procedure is not inherently precise. Silver particles do not attach to the target molecules in a fixed ratio and the aggregation of silver where concen-

trations of polysaccharide are high confound efforts to quantify experimental results. Despite these limitations we counted silver aggregates to estimate the protection afforded to polysaccharides by clay signatures and surfaces. Two or three representative photographs, i.e., typical among our set of photographs for levels of porosity and distribution of silver aggregates, were chosen for manual counting in each of four sample categories: (1) undigested and (2) digested laboratory-consolidated sediment and (3) undigested and (4) digested natural organo-clay fecal pellet. Silver aggregates were counted separately (a) both on and within 10 nm of clay platelets and (b) in areas more than 10 nm from any clay domain. The areas represented by (a) and (b) in each photograph were estimated separately and used to calculate the number of silver aggregates per unit area for each photograph. Areas of photographs showing opaque clay domains were not included in area estimates. The average number of silver aggregates per unit area was calculated to estimate silver/polysaccharide surviving digestion on or near clay surfaces or in open areas in both the laboratory-consolidated sediment and the organo-clay fecal pellet.

3. RESULTS

3.1. Laboratory-consolidated organo-clay sediment

The clay fabric signature of the laboratory consolidated sediment used for assessing chitin protection (Fig. 1) typically shows the swirl pattern (Bennett et al., 1977) and diffuse spacing of clay domains characteristic of remolded and/or sheared, reconsolidated sediment (Bohlke and Bennett, 1980). Sections were cut from within a subsample of consolidated clay, so all the apparent open areas in two-dimensional sections are actually pores. Those that are not bounded by illite within the bounds of the photograph are

considered “large pores” for the purpose of description and discussion. Initial porosity of the two samples with 10% total organic carbon was $n = 77.6\%$ ($e = 3.46$; where $n =$ porosity, $e =$ void ratio) with a water content of 133.7% (percent dry weight or ratio of grams of water to grams of dry solids times 100) before consolidation. Following consolidation under a final uniaxial load of 732 kPa (7.64 TSF) the porosity was reduced to 62% ($e = 1.61$) for sample number 1.3 and 54% ($e = 1.18$) for sample number 2.4 (Electronic Annex EA-1). During consolidation, porosity (n , percent volume of pores) decreases, sediment becomes more dense (compact), and pores change size and shape with increasing overburden stress. This has been observed for natural and laboratory-consolidated samples (Bennett et al., 1977, 1989, 2004). Lower porosity and smaller pore size inhibit diffusion and enzyme attack of OM present in the muddy sediment. The sediment microfabric and consolidation behavior appear to be largely controlled by the silt-sized, large illite domains (90% w/w). However, smectite (10% w/w), comprising much finer sized clay particles ($<2 \mu\text{m}$), appears to have a strong affinity for the illite domains. Smectite is identified as electron-translucent, fine particles forming a halo around the relatively electron dense illite domains (Figs. 2 and 3). Ransom et al. (1998b) suggested that marine OM has a strong affinity for smectite.

Chitin was visualized in the undigested sample associated with domains of illite and closely associated with platelets of smectite. Silver particles appear in aggregated patches and in chains where the chitin polymer extends in the plane of the section (Fig. 2). Although silver particles indicating the presence of chitin could be seen close to illite aggregates, illite is electron dense, limiting our ability to visualize directly illite–chitin associations. Silver particles and aggregates can be seen around and on smectite platelets indicating that chitin adsorbs to smectite (Figs. 2 and 3).



Fig. 1. Illite (90%) and smectite (10%) with 10% chitin. Note the swirled arrangement of clay domains (two representative domains are marked by opposing pairs of arrows). Scale bar = 500 nm.

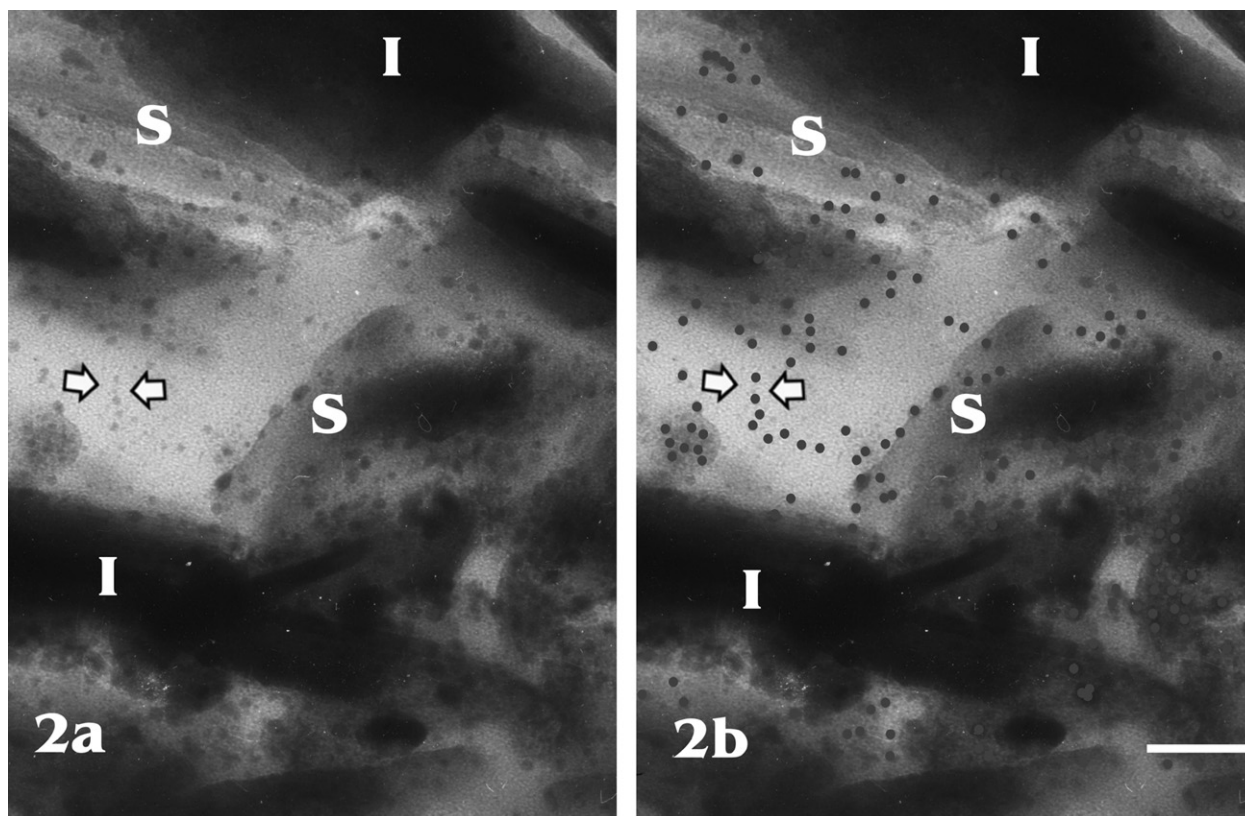


Fig. 2. Illite (90%) and smectite (10%) with 10% chitin undigested. (a) Relatively robust (cf. Fig. 3) silver aggregates indicate the location of chitin. Pair of opposed arrows bracket an orthogonal, linear chain of silver aggregates indicating a polymer of chitin in the plane of the section. (b) Same photograph with some of the silver aggregates marked with black dots for ease of interpretation. I, illite; S, smectite. Scale bar = 50 nm.

Patterns of silver aggregates indicated that chitin extended from clay platelets into large, open areas among clusters of clay domains, but chitin was less frequently seen unconnected to clay particles [consistent with “patches” of OM reported by Arnarson and Keil (2000), Mayer (1999), and Ransom et al. (1997)]. Pores and interstices <10 nm in diameter in the two-dimensional planes of our photographs contained little, or in most cases, no observable chitin in either the digested or undigested samples [consistent with Mayer et al. (2004)].

Chitinase digestion removed much of the chitin associated with pores (>10 nm; Fig. 3). Silver aggregates, indicating chitin distribution in Fig. 3, are sparse and tend to be obviously smaller and less numerous than in Fig. 2, which we interpret to result from enzymatic digestion. Silver aggregates were observed on smectite platelets indicating a high affinity of chitin for smectite (consistent with Ransom et al., 1998a). All these observations suggest protection by both adsorption and encapsulation by clay domains.

An exhaustive search of several samples (sections) representing undigested 10% chitin probed as described for controls 1 (no PA or TSC) and 2 (no TSC) showed no silver aggregates, indicating that silver proteinate does not bind nonspecifically to anything in the sample. The undigested 10% chitin sample treated as control 3 (no PA, but TSC ap-

plied) showed small amounts of silver comparable to the digested chitin sample indicating, in conjunction with controls 1 and 2, that some free aldehydes exist in the sample, presumably with the chitin, since it was the only organic material in the sample as determined by a probe of laboratory-consolidated sediment to which no chitin was added.

3.2. Natural organo-clay fecal pellet

The polychaete fecal pellet (Fig. 4) shows a much denser packing of clay domains at the surface of the pellet oriented in stepped face-to-face (stair-stepped or shingle) and edge-to-edge arrangements (Bennett et al., 1991) compared to the microfabric of the interior of the pellet. Within the pellet, we observed random arrangements of silver aggregates that might be encapsulated in three dimensions by microfabric signatures of randomly oriented domains. Some of these domains are at low-angle, edge-to-face contact that provides significant pore space for OM.

Silver particles and aggregates indicating the distribution of polysaccharide in the undigested fecal pellet sample were associated with clay particles and extended into open areas (Fig. 5). The digested material (Fig. 6) contained none of the large aggregates of silver seen in the undigested fecal

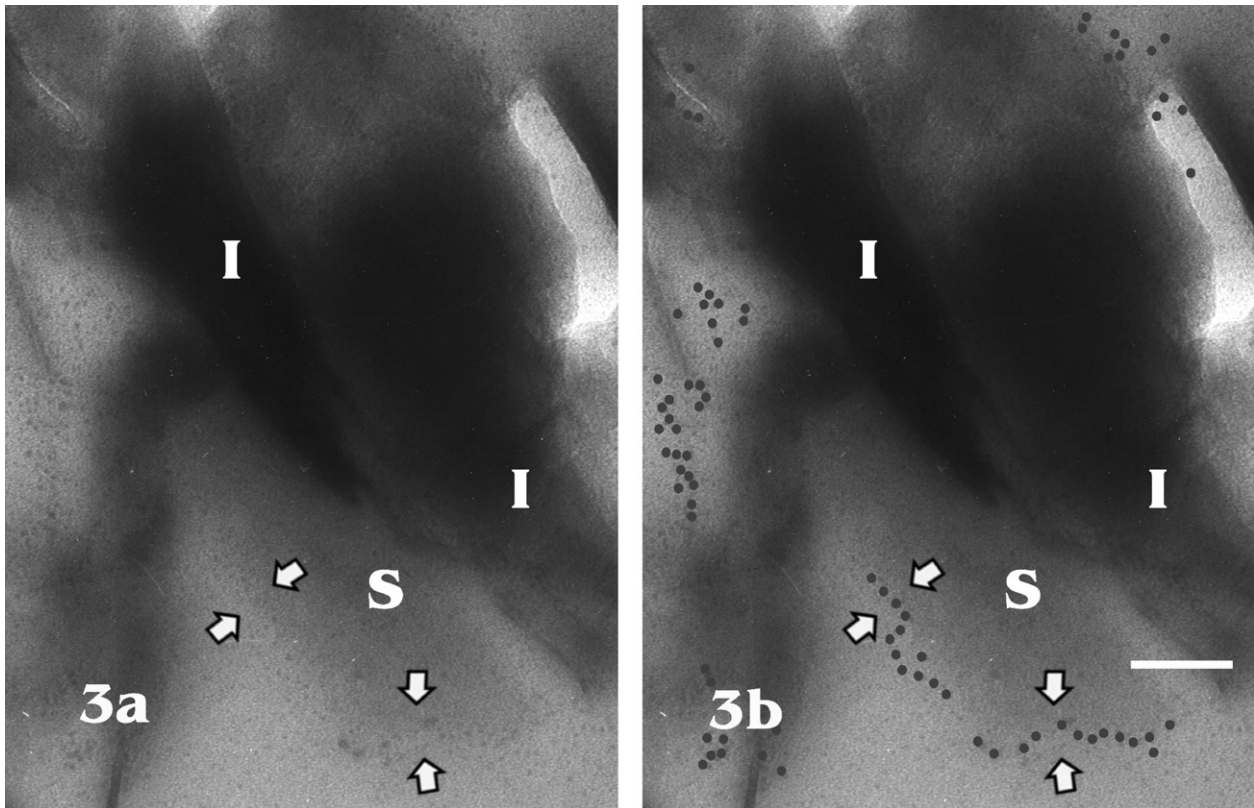


Fig. 3. Illite (90%) and smectite (10%) with 10% chitin after digestion with chitinase. (a) Fine silver aggregates indicate the location of chitin surviving enzymatic digestion. Particles are much finer than those representing undigested chitin (Fig. 2), indicating a degree of digestion. Pairs of opposed arrows bracket chitin on smectite. (b) Same photograph with some of the silver aggregates marked with black dots for ease of interpretation. I, illite; S, smectite. Scale bar = 50 nm.

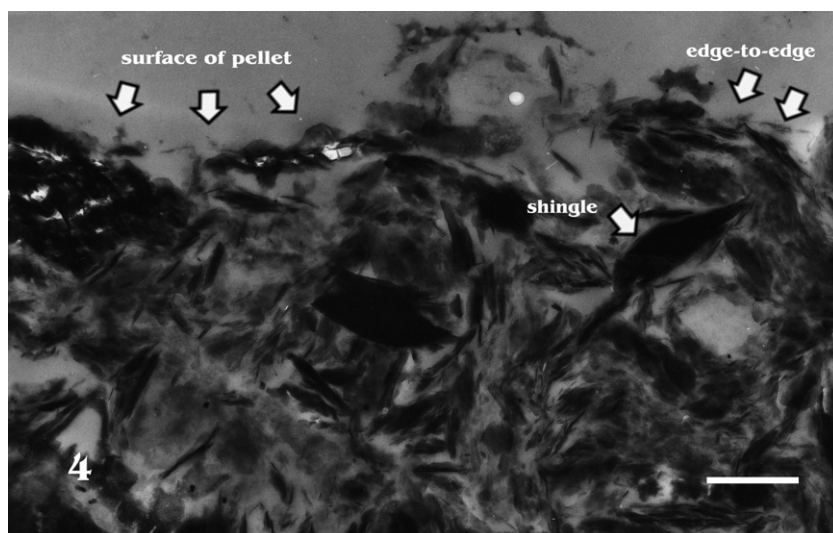


Fig. 4. Fecal pellet of *Heteromastus filiformis* sectioned at the surface of the pellet. Arrows point to representative edge-to-edge and shingle arrangements of clay domains. Scale bar = 500 nm.

pellet sample indicating a generally high degree of digestion. Silver/polysaccharide in the digested sample tended to be around clay, although it was occasionally observed

in open areas. This result is not surprising, since the fecal pellet was assumed to contain polysaccharides that were not hydrolyzed by the enzymes we used.

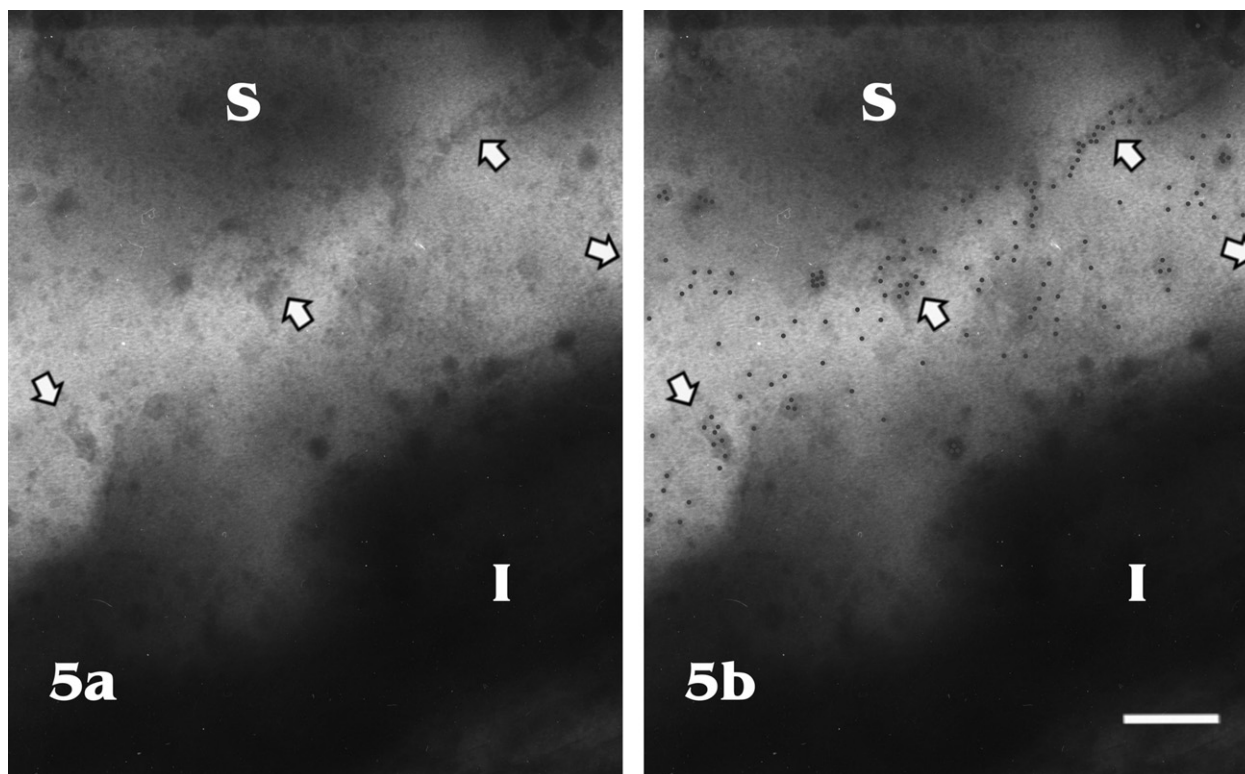


Fig. 5. Fecal pellet of *Heteromastus filiformis* sectioned near the surface of the pellet. (a) Fine silver aggregates indicate the location of polysaccharides that are widely distributed in the fabric of the pellet. Arrows point to a few selected patches of silver aggregates. (b) Same photograph with some of the silver aggregates marked with black dots for ease of interpretation. Scale bar = 50 nm.

3.3. Quantitative estimation of polysaccharides surviving enzymatic digestion

An estimation of the protective value of clay for OM indicated that 72% of the silver aggregates on and within 10 nm of the surfaces of clay platelets remained after digestion with chitinase in the laboratory-consolidated sediment (Table 2). Only 4% of the silver aggregates remained in areas more than 10 nm from any clay surface, i.e., pores.

A similar estimate for the organo-clay fecal pellet showed 48% of silver aggregates remaining in areas on and within 10 nm of a clay platelet surface after digestion with a mixture of five enzymes and 29% of the silver aggregates remaining in areas more than 10 nm from any clay surface (Table 2).

4. DISCUSSION

Many experiments have been conducted addressing the protection of OM by minerals to which they have been adsorbed. In most experimental protocols, it is impossible to differentiate how protection is achieved, because sorption to a surface (Hypothesis 1) may be accompanied by encapsulation within the fabric (Hypothesis 2). The operative principle is therefore obscured. Experiments comparing porous with nonporous mineral phases, which are otherwise chemically identical, provide a method to estimate the relative contributions of protection by sorption and

by encapsulation (Zimmerman et al., 2004). Direct visualization of substrate surviving enzymatic digestion, as employed here, is another.

The use of laboratory-consolidated sediment allowed us to control many variables, the most important of which was the OM substrate. The PA–TSC–SP procedure is general for a broad range of polysaccharides. Our artificial system contained only one polysaccharide, chitin, so the effect of enzymatic digestion was straightforward. We chose fecal pellets for natural sediment with the understanding that interpretation using this experimental system would be more difficult than interpretation using the laboratory-prepared samples. Polychaete fecal pellets are relatively easy to obtain, are rich in OM, and the peristaltic action of the worm alimentary canal reorients the outer “skin” of the pellet forming microfabric signatures that are likely to protect OM. Rhoads (1974) noted that virtually all of the surficial fine-grained marine sediments rich in OM have been processed through the guts of polychaetes and other deposit-feeding invertebrates. The rich variety of OM in a fecal pellet would probably contain polysaccharides that our mixture of five enzymes could not degrade; nevertheless, we were able to show a similar pattern of protection in both the laboratory system and the natural system.

The method we chose for visualizing the polysaccharide component of OM gave a relatively clear, qualitative and semi-quantitative picture of distribution within clay nano- and microfabric. The technique is less well suited for the

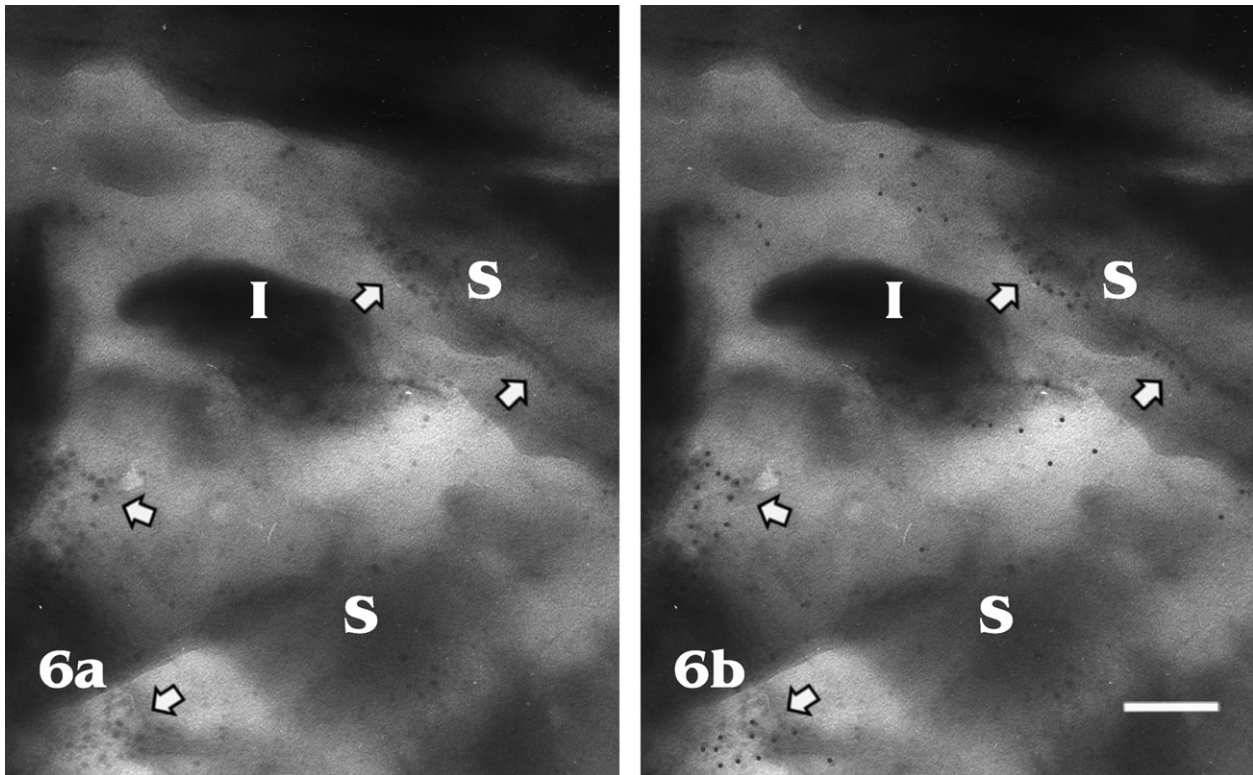


Fig. 6. Fecal pellet of *Heteromastus filiformis* sectioned near the surface of the pellet and digested with a mixture of chitinase, cellulase, α -amylase, pectinase, and dextranase. (a) Silver aggregates indicate the location of polysaccharide surviving enzymatic digestion. Arrows point to a few selected patches of silver aggregates mostly on the edge of smectite. (b) Same photograph with some of the silver aggregates marked with black dots for ease of interpretation. Scale bar = 50 nm.

quantitative analysis we attempted. The silver particles form aggregates, especially where polysaccharides concentrations are high. Counting the aggregates will give only an estimate of polysaccharide concentration. Nevertheless, this estimate showed that polysaccharides on clay surfaces in both laboratory and natural systems were less vulnerable to enzymatic degradation than those within pores (which in the photographs appear as open areas). We defined as large pores those apparently open areas more than 10 nm from any clay surface, choosing this dimension based upon the diameters of enzymes similar to those used (Table 1). Our main concern was to include interstices, as well as open clay surfaces, as protected areas. Surfaces should not be considered strictly two-dimensional for the purpose of OM protection. Organic matter attached to surfaces will extend away from them, and the extent to which this attached OM is protected can be understood through direct visualization.

Our laboratory-consolidated sediment showed a higher level of chitin protection than polysaccharide protection in the natural system. We attribute the higher level of overall protection in the laboratory sediment to the presence of its high smectite content. The natural system showed a higher level of polysaccharide surviving in open areas (pores) than was found for the laboratory system likely due to incomplete degradation of the wide variety of polysaccharides in a fecal pellet. The quantity of silver aggregates

remaining after digestion in the natural system was less than silver aggregates remaining in the laboratory system. We had several concerns about comparing the two systems that might explain the difference in silver aggregates left after digestion. Among those concerns, the laboratory system was pure clay sediment; the natural system contained fragments of diatom frustules and other materials that diminished the concentration of clay. We anticipate that the more complex fabric of the pellets would provide a better encapsulation of OM than the remolded laboratory system.

Despite these differences, we were able to demonstrate via experiments coupled to direct visualization the protective value of clay particles for OM. The pattern of enzymatic digestion in both systems indicated that the most complete digestion of polysaccharide was in the accessible pore volume (n_c). Polysaccharide remaining in the digested samples was largely confined to clay surfaces bordering large pores or to the small pores and interstices of clay domains. This pattern of survival is consistent with the two modes of clay protection that are commonly discussed—adsorption to clay surfaces (Hypothesis 1) and encapsulation (Hypothesis 2).

Hypothesis 1 is supported by the survival of surface-associated chitin in large pores that showed no chitin surviving within the interior of the pore, implying that attachment alone can play some role in protection. The potential

Table 2
Estimation of polysaccharide surviving enzymatic digestion based on silver particle counts per 10 nm² area of image

Laboratory-consolidated sediment	Ag particles/10 nm ²		Organo-clay fecal pellet	
	Near or on a clay surface ^a	Open area ^b	Near or on a clay surface ^a	Open area ^b
<i>Undigested</i>				
Photo 224098	0.56	0.43	0.28	0.10
Photo 224099	0.60	0.50	0.31	0.48
Mean	0.58	0.47	0.66	0.97
			0.42	0.52
<i>Digested</i>				
Photo 224137	0.31	0.01	0.18	0.12
Photo 224141	0.53	0.03	0.21	0.17
Mean	0.42	0.02	0.20	0.15
Ag particles after digestion	72%	4%	48%	29%

^a Silver aggregates on and within 10 nm of a clay platelet surface.

^b Silver aggregates more than 10 nm from clay.

^c Three photographs were used because of inherent high OM variation.

protective nature of clay surfaces is suggested by studies of enzymatic activity with substrates adsorbed onto mineral surfaces; such studies sometimes show decreased enzymatic activity that can be attributed to steric hindrance, adsorption of the enzyme to the mineral surface, or pH changes close to the mineral surface (Naidja et al., 2000; Zimmerman et al., 2004). In natural systems, Bennett et al. (1996) reported OM attached to clay particles and domains and Ransom et al. (1997) showed by direct observation with TEM a patchy distribution of OM on clay surfaces from several sampling locations. Nevertheless, adsorbed polymers can be hydrolyzed by adsorbed enzymes (McLaren and Estermann, 1956; Zimmerman et al., 2004), suggesting a unique combination of circumstances not evident in our investigation. Our direct observation indicates that enzymatic digestion of chitin is reduced at and near clay surfaces by comparison with open pore spaces.

Hypothesis 2 offers explanations of protection of OM by encapsulation within microfabric signatures (inaccessible porosity, n_i , the pore volume to which the enzymes cannot enter confined pore space) developed by clay domains. Sollins et al. (1996), Baldock and Skjemstad (2000), and Six et al. (2002) have discussed some forms/mechanisms of this type of protection. An encapsulation hypothesis does not necessarily include adsorption to a mineral surface as a mechanism of OM protection, but protection from enzymatic degradation is by enzyme exclusion rather than surface attachment per se. Six et al. (2002) suggested that encapsulation is more important than adsorption in soil systems. We observed silver aggregates/chitin in some areas completely enclosed by illite (pores up to ~700 nm) and in association with smectite within a given photograph, and in the samples subjected to digestion, interpreted them as an indication of physical protection by the microfabric formed by the clay domains. The upper value of pore size protection of ~700 nm that we observed is imposed by the level of organization and scale at which we were working and not by restrictions of clay microfabric. Enclosure in very small pores (<8 nm) that can directly exclude enzymes was possible, but the minor amount of polysaccharide found in such pores in this study prevents our testing for enzyme exclusion. Pore throats small enough to block entry of enzymes into large pore spaces containing OM may be a determining factor in OM protection (Mayer et al., 2004). Our TEM application, without reconstruction of serial sections, does not allow a good test of this possibility, because we could not view all of the potential pore throats that allow access to any visualized pore.

5. SUMMARY

Our results corroborate other studies suggesting OM protection from degradation and add the novelty of direct visualization. Observations with TEM demonstrate significant preservation of OM both by encapsulation and by adsorption in clay sediment. We emphasize that our results address protection on a fairly short time scale, providing indications of survival locations for polysaccharides that may be otherwise protected at longer time scales (Mayer,

2004). This study reports initially only on proposed protection of OM in muds equivalent in physical properties (water content and porosity) to recent natural surficial sediment on the order of thousands of years of age and less. Over long geological time-scales surficial sediment consolidates and loses water, often experiencing considerable reorientation and changes in the sediment fabric (Bennett et al., 1989). Future research opportunities could investigate the preservation of OM in relationship to fabric changes and sediment diagenesis during deep burial and large overburden consolidation.

This study advanced our understanding of the quantitative differences and significance in OM sequestering between bulk sediment porosity, n , effective porosity (n_e), and “inaccessible” porosity (n_i), where $n_i = (n - n_e)$. In this regard, we note that “inaccessible” porosity may indeed be filled with OM in different types of microfabric signatures and at different pore size scales. This is in contrast to effective porosity which corresponds to the sediment pore volume and pore fluid pathways that enables efficient convection (permeability), diffusivity processes, and mobility of enzymes. This research demonstrates that the term n_i is indeed an important quantitative and mechanistically different part of the total bulk porosity (Electronic Annex EA-1). Mechanisms of carbon protection may well be manifested in different ways at different levels of scale (nanometers, micrometers, and different fabric signatures such as aggregates, etc.) as we have demonstrated here.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gca.2007.01.009.

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