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Assessing biomarker syngeneity: An in situ approach using monoclonal antibodies

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ABSTRACT

Lipid biomarkers preserved in ancient rocks have the potential to reveal much about ancient ecosystems. However, establishing that the compounds of interest are syngenetic has proven to be an analytically challenging task. Traditional biomarker analyses rely on extraction of large quantities of powdered rock, making the association of molecules with sedimentary fabrics difficult, if not impossible. As an alternative approach, here we show that monoclonal antibodies that bind specifically to geolipids can be used as molecular probes for in situ detection and localization of such compounds. Monoclonal antibodies that bind to squalene and cross-react with the biomarker squalane were evaluated for labeling sediment-associated hydrocarbons. The anti-squalene antibodies were shown by dot immunoblotting with composed standards to cross-react also with other isoprenoids, such as phytol and its diagenetic products, suggesting reactivity towards acyclic isoprenoids. Then, the anti-squalene antibodies were shown to react with naturally occurring crude oils and, via an immunofluorescence-labeling approach, to bind to isolated organic-rich laminae in rocks from the Eocene Green River Formation known to contain squalane among other linear isoprenoids. These results suggest that squalane, or structurally similar organic biomarkers that cross-react with the antibodies, are confined to discrete organo-sedimentary fabrics within those rocks, providing evidence for their syngeneity. Depending on the specificity and sensitivity of the antibody/geolipid pair, an in situ antibody detection approach may be useful for establishing biomarker syngeneity in older rocks.

1. Introduction

Lipid biomarkers are organic compounds with hydrocarbon skeletons that can be related to biochemical precursors that are presently thought to be restricted to specific taxonomic groups (Brocks and Summons, 2003; Peters et al., 2005). Many lipid biomarkers are stable over geologic time, and the identification of these so-called geolipids in rocks and sediments has provided an alternative source of evidence for the appearance of specific taxa in the rock record (Moldowan and Talyzina, 1998; Brocks et al., 1999; Brocks and Summons, 2003; Brocks and Pearson, 2005). However, biomarker studies are commonly complicated by the possibility of contamination, which introduces non-syngenetic biomarkers to the host rocks (Brocks, 2011; French et al., 2015). Here we propose the utilization of monoclonal antibodies as molecular probes to visualize specific biomarkers in situ and address syngeneity by relating their spatial distribution to the sedimentary fabric of the host rock.

1.1. Biomarker syngeneity

A biomarker is taken to be syngenetic if it was introduced from the primary depositional environment. However, the mobilization of petroleum can introduce non-indigenous hydrocarbons into sedimentary rocks along the pathway of migration (Curiale and

Abbreviations: BSA, bovine serum albumin; CN, 4-chloro-1-naphthol; DAB, 3,3-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IgM, immunoglobulin M; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TBS, tris-buffered saline; TOF-SIMS, time of flight secondary ion mass spectrometry.

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Bromley, 1996). In addition, anthropogenic petroleum products can contaminate samples during collection and storage (Hoering, 1965, 1966). For these reasons, establishing the syngeneity of geolipids is a critical prerequisite to their use as paleobiological indicators (Meinschein, 1965; Nagy, 1970; Brocks et al., 2003). Brocks et al. (2003) presented a number of analytical approaches to provide evidence of biomarker syngeneity in Archean rocks. Such approaches include matching molecular indicators of thermal maturity with the age and lithological maturity of the host rocks, comparing the stable isotope values of sample kerogen and bitumen, assessing the geological context of the host rocks for potential contamination by migrating hydrocarbons, evaluating the spatial distribution of hydrocarbons within the sample and analyzing the pyrolytic degradation products from kerogen. However, these approaches are not always possible, nor are their results fully conclusive (French et al., 2015).

One alternative approach to assessing biomarker syngeneity would be to evaluate the association between specific organic biomarkers and primary or secondary organo-sedimentary fabrics, as indicators of endemicity or contamination, respectively. In this paper, we show that the distribution of specific compounds within rock fabrics may, in certain cases, be visualized through a new in situ detection method using monoclonal antibodies that bind to specific classes of geolipids (or to geolipids in a broad sense). Association of the biomarkers with the primary sedimentary fabric (e.g., organic rich laminae vs secondary veins) would provide additional evidence for the syngeneity of antibody-binding hydrocarbons.

1.2. Antibodies

The immune system of vertebrates uses antibodies as part of an adaptive response to recognize and disable potentially harmful compounds and pathogens (e.g., Abbas et al., 1991; Roitt, 1991; Birch and Lennox, 1995). Because antibodies bind to specific sites (epitopes) on the target molecules (antigens), custom antibodies are commonly developed and used as probes for the detection of specific molecules in modern life science applications, ranging from cancer treatment (Berinstein and Levy, 1987; Scheinberg, 1991; Biela et al., 2003) to microbial ecology (Lin and Carpenter, 1996; Faude and Höfle, 1997; Caron et al., 2003). Immunodetection approaches have even been used in paleontological studies. For example, Muyzer and Westbroek (1989) raised polyclonal antibodies against the organic matrix of recent bivalve shells. The antibodies also reacted with biopolymers preserved in Pleistocene bivalve shells suggesting a chemical similarity between the modern and ancient shell matrices (Muyzer et al., 1984; Muyzer and Westbroek, 1989; Collins et al., 1991, 2003).

Antibodies commonly bind to epitopes with remarkable selectivity. When used in immunolabeling applications, usually a secondary antibody that is conjugated to a detection probe is added. The secondary antibody is designed to bind the primary antibody, allowing the antibody-antigen interaction to be detected, either in situ or in an extract (Blanchette-Mackie et al., 1989; Compère et al., 1995). Biological antigens are typically proteins and polysaccharides that have epitopes derived from their complex functional groups and three-dimensional stereochemistry (Liddell and Cryer, 1991; Li et al., 1998). Although lipids, especially in their saturated hydrocarbon form, are structurally simple molecules compared to other antigenic biomolecules, antibodies that exhibit specific binding to lipids are well documented (Fogler et al., 1987; Swartz et al., 1988; Maneta-Peyret et al., 1992). For example, human blood is known to contain antibodies against cholesterol, which is thought to act as a mechanism for regulating low-density lipoprotein production (Swartz et al., 1988; Alving et al., 1989; Alving and Swartz, 1991). Naturally occurring antibodies, however, likely bind epitopes containing polar groups, such as the hydroxyl group on cholesterol. Antibodies binding hydrophobic portions of lipids are less likely to occur and therefore the immunological study of biomarkers in sedimentary rocks has been poorly documented thus far.

1.3. Antibodies to target lipid biomarkers

Maule et al. (2003) produced a polyclonal antibody serum that showed some reactivity to hopanes for use as a stand-alone biomarker detection tool for potential life on Mars. In the context of Mars exploration, other attempts have been made to produce polyclonal antibodies in the development of the Life Marker Chip, an antibody-based tool to detect small hydrophobic molecules in situ, such as the lipid biomarkers coprostane, hopane, phytane and squalane among others (Rix et al., 2011; Sathe et al., 2011; Sephton et al., 2013). However, the heterogeneous nature of polyclonal antibodies, which consist of a mixture of multiple antibodies that target different epitopes and potentially different molecules, fundamentally limits confidence in their capability to bind only to the target compound in complex molecular assemblages of structurally similar hydrocarbons, such as those found in sedimentary bitumen and petroleum. In addition, polyclonal antibodies would likely bind to a polar functional groups not usually found in sedimentary biomarkers.

More promisingly for studies of natural organic matter, Matyas et al. (2000, 2002) produced several mouse monoclonal antibodies against the lipid squalene. In contrast to polyclonal antibodies, monoclonal antibodies derive from an isolated clone of a single antibody-producing cell. Therefore, the antibodies that bind to polar moieties on biomarker structures can be selected from the population of antibodies comprising the immune response. Among the antibody-producing clones obtained by Matyas et al. (2000), the hybridoma[3] SQE#14 (PTA6538) secreted antibodies shown by ELISA not only to bind squalene, but also to bind to its saturated form, squalane, a relatively common sedimentary geolipid (Matyas et al., 2000, 2002; Alving et al., 2005).

Squalene is the biochemical precursor for the synthesis of all polycyclic triterpenes and sterols. Therefore, squalene is synthesized by all domains of life and its saturated analog, squalane, is ubiquitously found in sediments and known to occur in ancient rocks as a sedimentary hydrocarbon (Gardner and Whitehead, 1972; Peters et al., 2005). The cross-reactivity of the antibodies from the clone SQE#14 was exploited in this study to detect sedimentary squalane (or other hydrocarbons the antibody may bind to) and test the ability of monoclonal antibodies to target lipid biomarkers in the rock record. In this study, the antibodies produced by Matyas et al. (2000) were evaluated for their molecular specificity by measuring their binding affinity, first to standard solutions and crude oil samples containing other common sedimentary hydrocarbons to test the feasibility of the antibody-geolipid binding. Then, as a test case, anti-squalene antibodies were applied to squalane-containing mudstones from the Eocene Green River Formation for biomarker visualization in rock-associated sedimentary organic matter.

2. Materials and methods

2.1. Hybridoma culturing and antibody production

Hybridoma clone PTA6538-SQE14 cells were obtained from the American Type Culture Collection and were cultured in hybridoma

[3] A hybrid cell line obtained by the fusion of an immortal myeloma cell line and the antibody-producing cells from the spleen of the mouse that was injected with the desired antigen. The hybridoma can be cultured in vitro indefinitely and, when isolating a particular cell clone, the same specific antibodies can be collected from the culture media.

growth medium (GIBCO; Formula number 06-5012EL supplemented with 3% characterized fetal calf serum). Cells were grown initially in T75 flasks. When confluent, the cells were further expanded to T250 flasks and then added to 3 L spin flasks. When cells reached a concentration of 1×10^6 cell/mL, the flasks were aerated for an additional 3–5 days of growth. After cultivation, the cell suspension was poured into 500 mL conical centrifuge tubes and spun at 2400 rpm for 15 min at 4 °C. The supernatant was then filtered through a 0.2 μm 1 L bottle top filter unit into a 5 L sterile glass bottle. Several aliquots of the filtered supernatant were stored at −20 °C until they were thawed and used as the primary antibody. Anti-CD75 IgM antibodies from the clone LN-1, which react with the human surface proteinaceous lymphocyte antigen CD75, were similarly obtained from culture supernatants and used as an irrelevant isotype-control antibody in all immunolabeling experiments.

2.2. Immunoblotting on PVDF membranes

Biomarker standards and crude oil samples were evaluated for their binding affinity to the antibodies by dot immunoblotting (Stott, 2000). The isoprenoids squalene, phytane, stigmasterol (24-ethylcholesta-5,22E-dien-3β-ol), phytol (Sigma-Aldrich, St. Louis, Missouri, USA), squalane, pristane and cholestane (Acros Organics, Geel, Belgium) together with the alkanes *n*-pentadecane, *n*-icosane and *n*-tetracosane (Alfa Aesar, Ward Hill, Massachusetts, USA) were used as biomarker standards. The crude oil samples (ONTA Inc., Toronto, Ontario, Canada) were numbered from one to eight in increasing order of density, corresponding to: 1, paraffinic oil from the Appalachian Basin, USA (827 mg/mL); 2, paraffinic oil from Louisiana, USA (839 mg/mL); 3, paraffinic oil from Qua Iboe, Nigeria (846 mg/mL); 4, paraffinic-naphthenic oil from Edmonton, Alberta, Canada (861 mg/mL); 5, naphthenic oil from Hoops, Texas, USA (869 mg/mL); 6, aromatic-intermediate oil from Saudi Arabia (870 mg/mL); 7, aromatic-asphaltic oil from Oriente, Ecuador (910 mg/mL); 8, aromatic-asphaltic oil from Merey, Venezuela (968 mg/mL).

One microliter of biomarker or crude oil solution dissolved in *n*-hexane (with the exception of stigmasterol, which was dissolved in tetrahydrofuran; Sigma-Aldrich) at different concentrations were spotted onto 0.2 μm PVDF membranes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) without previous activation of the membrane with alcohol. The biomarker-coated membranes were dried overnight at room temperature and then incubated for one hour under orbital shaking at 25 °C with 2% bovine serum albumin (BSA; Fraction V from Roche Diagnostics GmbH, Mannheim, Germany) dissolved in TBS solution (Tris-buffered saline, 20 mM Tris Base pH 7.6, 150 mM NaCl). Membranes were then incubated for one hour under orbital shaking at 25 °C with undiluted culture supernatants of either the hybridoma clone SQE#14 (PTA6538) or the hybridoma clone LN-1, both cell lines that secrete mouse antibodies of the isotype IgM. After the primary antibody incubations, the membranes were washed three times for 15 min each under orbital shaking at 25 °C with TBS and then incubated for one hour under orbital shaking at 25 °C with goat anti-mouse IgM horseradish peroxidase-conjugated antibodies (Invitrogen, Waltham, Massachusetts, USA) diluted 1:1000 in TBS. The membranes were subsequently washed three times for 15 min each under orbital shaking at 25 °C with TBS and the secondary antibody binding was revealed by a chromogenic precipitate developed after the addition of CN/DAB (4-chloro-1-naphthol/3,3′-diaminobenzidine) substrate, according to the manufacturer's instructions (Pierce™ CN/DAB substrate kit, Thermo Fisher Scientific).

It is important to note that because the biomarkers used to coat the membranes are hydrophobic, they are susceptible to diffusion after coating and may elute from PVDF membranes if: (1) the membranes are pre-activated by immersion in alcohol and maintained wet prior to antigen spotting, as is typically required for activation of PVDF membranes when proteins are blotted, or (2) the membranes are washed with solutions containing detergent-like solvents, such as polysorbate 20 or other non-ionic detergents that are frequently used to reduce non-specific antibody interactions in immunoblotting applications (Alving and Grabenstein, 2000). Therefore, no pre-activation or washing solutions containing detergents were used here to avoid diffusion and removal of the antigens spotted on PVDF membranes.

ImageJ (Schneider et al., 2012) was used to subtract the background of the dot blot images and quantify the chromogen reciprocal intensities (Nguyen and Nguyen, 2013). Each dot intensity obtained was normalized to the intensity of the 2 μL dot of anti-CD75 culture supernatant diluted 1:4 spotted together with the samples on every membrane. For comparison among biomarker standards and crude oil samples, the normalized intensities obtained by anti-squalene blotting were subtracted from the normalized intensities of the corresponding dot obtained by anti-CD75 blotting (isotype control) and the values were expressed as arbitrary units in Figs. 1 and 2. Graphs and statistical analyses between experimental values (anti-squalene) and the isotype control (anti-CD75) were performed using the Mann-Whitney *U* test on GraphPad Prism 5.0.

2.3. Immunofluorescence of organic matter in natural rock samples

Unweathered hand samples of lacustrine kerogen-rich mudstones from the Green River Formation, outcropping in Fossil Basin, were collected from a quarry near Kemmerer, Wyoming (Lat. 41.798; Lon. −110.732, Supplementary Fig. S1) and wrapped in pre-combusted aluminum foil for transport and storage. Rock samples were slabbed with a water-cooled unlubricated rock saw. One face was used for immunofluorescence and the other for conventional GC–MS analysis (see Section 2.4). Press-on immunoreagent wells were applied to the slab surfaces to keep control reagents from mixing with experimental reagents. Slab surfaces used for immunofluorescence were etched in 0.2% HCl before being rinsed three times with Milli-Q water. The acid treatment removes carbonates potentially blocking organic remains, therefore increasing the exposure of organic antigenic surfaces to antibodies. Rock surfaces were subsequently blocked with 2% BSA/PBS (bovine serum albumin dissolved in phosphate-buffered saline) and allowed to incubate in a humid chamber to prevent dehydration. Blocking solution was aspirated before 60 μL of antibody-containing supernatant diluted 1:10 in 2% BSA/PBS was added to each well and allowed to incubate in a humid chamber for one hour at ~25 °C. Upon aspiration, each well was washed ten times with 260 μL drops of 2% BSA/PBS with aspiration between washes. Twenty μL of secondary goat anti-mouse IgM FITC-conjugated antibody-containing solution diluted 1:1000 in 2% BSA/PBS was then added to each well and allowed to incubate in the dark for 1 h. After 10 washings with 20 μL drops of 2% BSA/PBS, 20 μL of VectaShield antifade mounting medium (Vector Laboratories Inc., Burlingame, California, USA) was added and a coverslip attached. FITC-conjugated antibodies were visualized using a Zeiss Axioscope equipped with Zeiss Filter Set 09 (BP) 450–490, (BMS) 510, (LP) 515, Zeiss, Jena, Germany. Image post processing was restricted to uniform whole-image level adjustments.

Two types of controls, with two replicate wells for each control, were contained on each immunofluorescence slide. First, all reagents were applied to un-etched portions of each section. Second, etched portions of the slide received a control treatment identical to the experimental treatment, but with an irrelevant primary antibody (anti-CD75, described in Section 2.1).

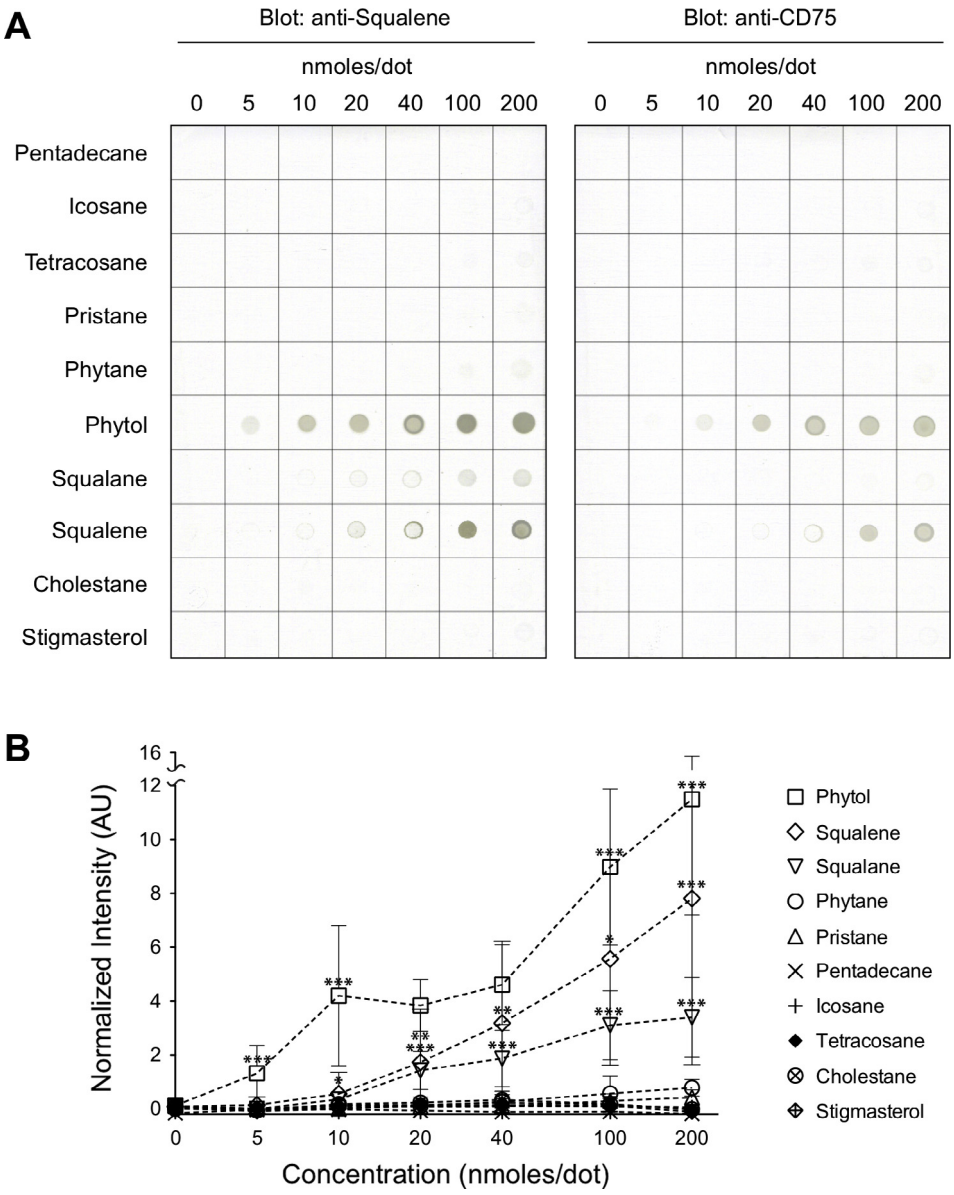

Fig. 1. Immunoblotting of biomarker standards on PVDF membranes: (A) Representative dot blot of biomarker-coated PVDF membranes using anti-squalene antibodies (left) and anti-CD75 antibodies as isotype control (right). The *n*-alkanes pentadecane, icosane and tetracosane, the acyclic isoprenoids pristane, phytane, phytol, squalane and squalene, along with the sterols cholestane and stigmasterol were used to coat the membranes at different concentrations; (B) semi-quantification of the dot blots shown in A. Data are expressed as arbitrary units (AU) of the mean of quintuplicate pixel intensities of each anti-squalene dot normalized with respect to an IgM antibody-coated dot and subtracted to the corresponding anti-CD75 normalized pixel intensities. Significantly higher values that are at least twofold above the background signal are indicated (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$).

2.4. Gas chromatography–mass spectrometry (GC–MS)

For this study, cuttings from the same hand sample used in the immunofluorescence detection experiment were powdered and their solvent extracts were analyzed by GC–MS to provide independent confirmation of squalane in the samples used for immunofluorescence. Powdered rock was extracted using dichloromethane. The saturate fraction was isolated by silica gel chromatography using *n*-hexane as a mobile phase. A branched/cyclic fraction was obtained using silicalite (ZSM-5) chromatography with isooctane as a mobile phase. GC–MS analysis of the rock extract was performed on a VG Autospec-Q mass spectrometer coupled to an HP Series II gas chromatograph fitted with an Agilent DB-1 silica capillary column (60 m long, 0.25 mm ID, 0.25 µm film thickness). The temperature program started with 150 °C for one min, followed by an increase of two degrees per min until 320 °C was reached and held for an additional 20 min.

Crude oil sample #2 (described in Section 2.2) was evaluated by selected ion monitoring (SIM) GC–MS on an Agilent 5975c MSD with a 7890A GC front end coupled to an Agilent DB-1 silica capillary column (60 m long, 0.25 mm i.d., 0.25 µm film thickness). The chromatographic temperature program started at 35 °C, increasing at 2 °C/min until 80 °C was reached, followed by an additional increase of 3 °C/min until 320 °C was reached and held for 15 min. The quantification of squalane was calibrated with an internal standard of squalane and detected using the *m*/*z* 113 ion. Other acyclic isoprenoids were also identified in the analysis and their approximate concentrations were calculated using the *m*/*z* 113 ion, assuming the same sensitivity response as squalane (Supplementary Table S1).

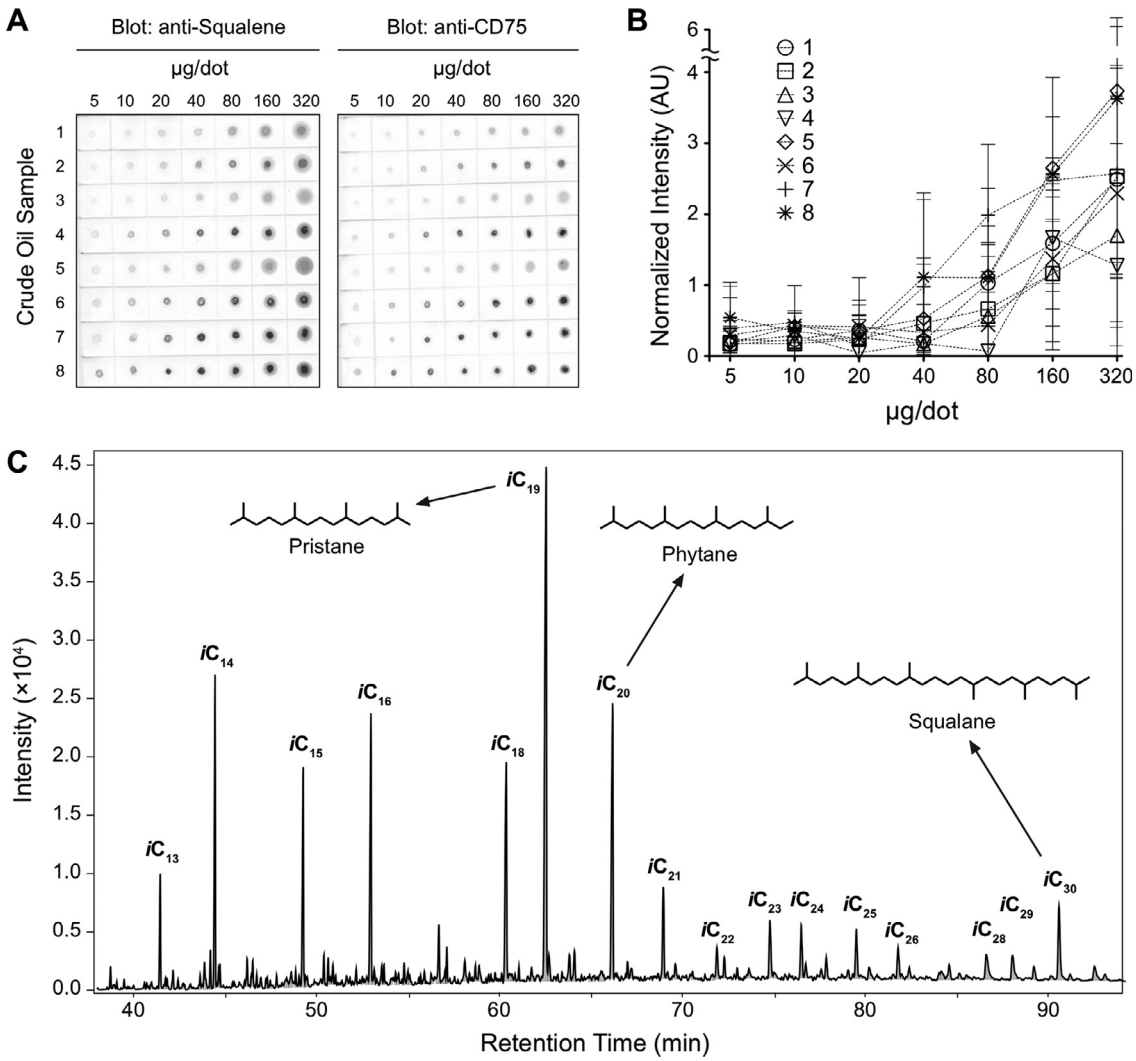

Fig. 2. Immunoblotting of crude oil samples on PVDF membranes: (A) representative dot blot of crude oil samples used to coat PVDF membranes that were incubated with anti-squalene antibodies (left) and anti-CD75 antibodies as isotype control (right). Membranes were coated with different concentrations of eight crude oil samples: 1, paraffinic oil from the Appalachian Basin, USA; 2, paraffinic oil from Louisiana, USA; 3, paraffinic oil from Qua Iboe, Nigeria; 4, paraffinic-naphthenic oil from Edmonton, Alberta, Canada; 5, naphthenic oil from Hoops, Texas, USA; 6, aromatic-intermediate oil from Saudi Arabia; 7, aromatic-asphaltic oil from Oriente, Ecuador; 8, aromatic-asphaltic oil from Merey, Venezuela; (B) semi-quantification of the dot blots shown in A. Data are expressed as arbitrary units (AU) of the mean of duplicate pixel intensities corresponding to each dot normalized with respect to an IgM antibody-coated dot and subtracted to the anti-CD75 normalized pixel intensity. (C) Mass chromatogram of *m*/*z* 113 of the oil sample 2, showing the identification of squalane by GC–MS at concentrations of 516 mg/L (0.6 mg/g). Other isoprenoids were also identified using the *m*/*z* 113 ion as shown in the chromatogram.

3. Results

3.1. Evaluation of anti-squalene antibody specificity

Squalene, an acyclic terpenoid composed of six isoprene subunits (triterpene, C_{30}), was used as immunization antigen by Matyas et al. (2000) for the production of several antibody-secreting hybridomas. One of the obtained hybridomas, the clone SQE#14 (PTA6538), secretes antibodies that have been previously shown by ELISA to also bind squalane (Matyas et al., 2000). The binding promiscuity of these antibodies may be useful for sedimentary hydrocarbon detection, because it is possible that the antibodies bind to other biomarkers along with squalane and squalene. We tested the cross-reactivity of hybridoma supernatants from the clone SQE#14 against other relevant lipid biomarkers that are structurally similar to squalene (see Supplementary Fig. S2 for a structural comparison of the hydrocarbons used in this study).

Antibody binding reactivity was evaluated by an immunoblotting procedure adapted to detect biomarker standards by dot blot on PVDF membranes. Standard solutions of the acyclic isoprenoids squalane (C_{30}), squalene (C_{30}), phytol (C_{20}), phytane (C_{20}) and pristane (C_{19}) along with the cyclic isoprenoids cholestane (C_{27}) and stigmasterol (C_{29}) as well as the alkanes *n*-pentadecane (C_{15}), *n*-icosane (C_{20}) and *n*-tetracosane (C_{24}) were used to coat PVDF membranes with one microliter at concentrations ranging from 5 to 200 nmoles/µL. After anti-squalene antibody incubation, binding was detected by the addition of peroxidase-conjugated anti-IgM antibodies and CN/DAB substrate, which forms a blackish precipitate on the membrane (Fig. 1A).

In addition to squalene and squalane, the antibody derived from the clone SQE#14 displays binding affinity to phytol and, to a lesser extent, phytane and pristane (Fig. 1A). Immunoblotting using an irrelevant IgM antibody (anti-CD75) is also shown in Fig. 1A to reveal the background signal intensity derived from the lipid coatings, non-enzymatic reaction and potential non-specific binding of both primary and secondary antibodies. Particularly for phytol and squalene, the background developed was significantly higher than for other lipids. To compare the signal intensities among each

hydrocarbon tested, the anti-CD75 control signal was subtracted from the anti-squalene intensities, so the semi-quantitative results in Fig. 1B reflect specific binding of the antibodies from the clone SQE#14. By comparing to the background intensities, the anti-squalene antibody provides significantly higher values for phytol starting at 5 nmoles/dot[4] ($p < 0.01$), squalene at 10 nmoles/dot ($p < 0.1$) and squalane at 20 nmoles/dot ($p < 0.01$; Fig. 1B). Considering the statistical significance and at least a twofold intensity of the experimental condition over the background signal, the detection limit for squalane ranges from 10 to 20 nmoles/dot (1.2 to 2.4 nmoles/mm^2) under the tested conditions. Phytane and, to a lesser extent, pristane show significant increase of intensities over 100 nmoles/dot; however, their values remain comparatively low and do not rise twofold above the background. The immunoblottings also show no twofold increase above the background and no significantly higher intensities for coatings of the *n*-alkanes and the cyclic isoprenoids (Fig. 1B). Even at higher hydrocarbon concentrations of 2000 nmoles/dot, the alkanes and cyclic isoprenoids tested showed no apparent signal increase (data not shown), suggesting binding affinity to linear isoprenoids mostly.

3.2. Biomarker detection in crude oils by immunoblotting

In addition to assessing the specificity of anti-squalene antibodies to bind biomarker standards, immunoblotting of crude oil samples was also applied to evaluate the binding capacity of the antibody in a complex mixture of biomarkers. Eight crude oil samples from different field locations were spotted on PVDF membranes at increasing concentrations from five to 320 μg per dot. On their own, the crude oil membrane coats appear as a blackish-brown color. Therefore, to assess the binding affinity of anti-squalene antibody by immunoblotting, the color intensities were compared to PVDF membranes incubated with the anti-CD75 isotype control antibody, as described above (Fig. 2A).

Quantification of immunoblot signal intensities shows increasing tendencies towards higher concentrations for all crude oil samples analyzed, indicating greater intensity of the anti-squalene antibody signal compared to the isotype control (Fig. 2B). Analysis of the crude oil sample #2 by GC–MS indicates the presence of several isoprenoids along with squalane (Fig. 2C and Supplementary Table S1). The more abundant isoprenoids correspond to pristane (iC_{19}) and phytane (iC_{20}) with estimated concentrations of 2630 and 1250 μg/mL (3.1 and 1.5 mg/g), respectively. Squalane, although not as abundant as pristane and phytane, was found at concentrations of 516 μg/mL (0.6 mg/g). Therefore, a maximum of 3.7, 1.7 and 0.5 nmoles of pristane, phytane and squalane, respectively, were spotted per dot on the membrane for the crude oil sample #2, which are below the detection limits shown in Fig. 1. The anti-squalene antibody seems not only to bind squalane, but likely binds other isoprenoids. The cumulative effect of other acyclic isoprenoids (and perhaps other hydrocarbons not tested in this study) may explain the signal in Fig. 2A.

3.3. In situ detection in kerogen-rich marlstone using immunofluorescence

Rock sections from the Eocene Green River Formation were examined using petrographic and fluorescence microscopy after acid etching and antibody incubations. The examined rock sections consisted of kerogen-rich laminated mudstones characterized by alternating calcite-rich and kerogen-rich laminae. The mineralogy of the examined rocks consists primarily of calcite, with minor amounts of dolomite, quartz, feldspar, and clay, as previously reported by Buchheim (1994) and Buchheim and Eugster (1998). Kerogen occurs primarily as continuous, wavy organic-rich laminae, ~10–50 μm thick. Many of the laminae in the sampled rocks are discontinuous and evidence for soft-sediment deformation is present in hand samples.

In the rock sections, anti-squalene antibody binding was visualized by the addition of fluorescent FITC-conjugated anti-IgM antibodies followed by fluorescence microscopy. Of the more than 1100 individual laminae examined, only 14 total regions showed extensive antibody binding. These segments of antibody binding accumulation present as convolute undulations within the organic lamina, connecting small irregularly-shaped signal "patches" ~5 μm in diameter (Fig. 3A–C, Arrows). In total, antibody binding was observed in fourteen individual spots – although in all but three cases these occurred as small patches rather than single horizons that followed bedding several hundred microns in length. Antibody binding was not observed in control wells that examined subjacent portions of the same laminae containing un-etched surfaces (Fig. 3D). Examination of the control wells in which an irrelevant primary antibody was used on the same rock coupons as the test wells, also revealed no FITC signal (Fig. 3E).

Carbonate minerals within the marls tested commonly exhibited a dull greenish-yellow background autofluorescence with rare isolated grains showing bright yellow autofluorescence with diffuse margins (Fig. 3F). Kerogenous laminae and organic macerals such as microfossils were not autofluorescent. The yellow autofluorescence of the minerals and the green FITC antibody label showed spectral overlap that was clearly differentiated by visualizing the color signal under a FITC long-pass filter. In addition, the FITC probe showed distinctive sharp margins and photobleached during observation, whereas the mineral autofluorescence did not. Therefore, the green fluorescence within organic-rich laminae of acid etched surfaces incubated with anti-squalene antibodies is attributable to antibody binding. A variety of linear isoprenoids contained in the organic laminae may also bind the anti-squalene antibody, specifically pristane and phytane, which have been shown to dominate in Green River Formation Rocks (Eglinton et al., 1966; Robinson, 1979; Olcott Marshall and Cestari, 2015). Squalane, less studied in Green River samples, was evaluated by GC–MS performed on sample cuttings removed from the same slabs used for the immunofluorescence analysis. Supplementary Fig. S3 shows the chromatogram indicating the presence of squalane in extracts from the cuttings at concentrations of ~100 ppb (0.1 μg/g) as well as other acyclic isoprenoids found, such as pristane and phytane.

4. Discussion

4.1. Antibody binding specificity

The potential for antibodies to be used as probes for specific biomarker compounds (or structurally related classes of biomarkers) in ancient rocks is directly related to the antibodies' binding affinity for the target compound, as compared to other hydrocarbons that are also common in extracts from ancient organic matter. The antibodies used in this study, secreted by the hybridoma clone SQE#14, were not originally intended to bind biomarkers, yet their capacity to bind squalane and other geolipids could be useful for analyzing biomarker syngeneity in rock extracts.

The immunoblotting assay in Fig. 1 shows the relatively strong binding affinity of the anti-squalene antibody to some biomarkers, as compared with the weak signal observed in the isotype control. This background signal is prominent for the standards of squalene

[4] For convenience, lipid concentrations on the membrane are expressed in nmoles per dot. Each dot area was lipid- and concentration-dependent, with an average phytol dot of 11.1 ± 0.2 mm^2, squalane dot of 8.5 ± 0.03 mm^2 and squalene dot of 8.8 ± 0.05 mm^2.

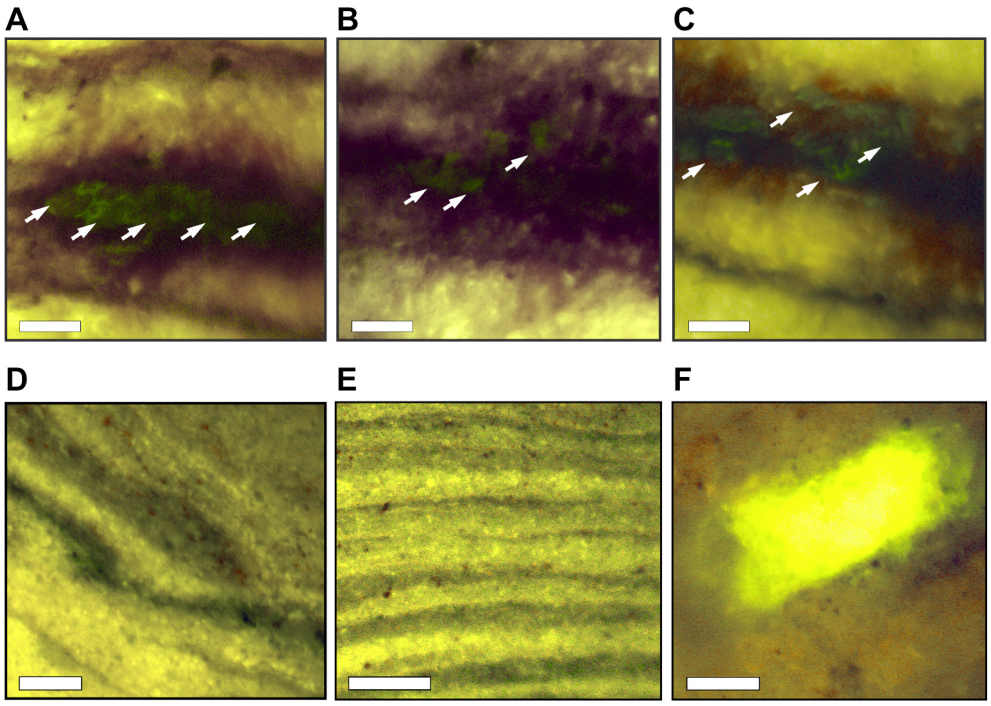

Fig. 3. Immunofluorescence of organic laminae from the Green River Formation: (A–C) Imaging of FITC-conjugated antibodies on three different sections of organic-rich slabs that were incubated with anti-squalene antibodies revealing discrete signal accumulation (arrows) and suggesting the presence of squalane/isoprenoids associated with individual laminae. Negative controls of acid un-etched sections incubated with anti-squalene (D) and acid-etched sections incubated with an irrelevant anti-CD75 primary antibody (E) did not exhibit an immunofluorescence signal. Mineral autofluorescence was present in the imaged sections as a dull yellow background and diffuse bright yellow associated with certain mineral grains (F). Autofluorescence is distinct from the sharply-localized green fluorescence of antibodies shown in A–C. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and phytol, and it is a contribution of the intrinsic opacity of each hydrocarbon coating over the PVDF membrane, the non-enzymatic reaction of the CN/DAB substrate with the lipids and non-specific molecular interaction of the different components of the supernatant medium along with non-specific binding of primary and secondary antibodies with the lipids. Among the above-mentioned factors, non-specific binding of the primary antibody is the most sensitive problem, because it may give false positive signals in complex matrices. In this study, non-specific binding may be produced because of the mild conditions used in the incubation and washing steps during the immunoblotting that involve the usage of non-detergent containing solutions (see Section 2.2). Nonionic surfactants are usually incorporated in the washing solutions to avoid non-specific hydrophobic interactions between proteins; however, they would also remove the lipids used in this study and the immunoblotting results would not be reproducible (Alving and Grabenstein, 2000). Therefore an isotype control should be carried out on each blotting procedure to compare binding affinities and acknowledge false positive signals, as shown in Fig. 1A.

Even though the isotype control signal was subtracted from the anti-squalene, cross-reactivity of the anti-squalene antibodies against phytol was observed. The quantified binding intensities for phytol are comparable and even higher than the intensities of the cognate antigen, squalene, suggesting binding multiplicity (Fig. 1B). It is well recognized that proteins, among them enzymes and antibodies, can present functional promiscuity (James and Tawfik, 2003). In the case of antibodies, promiscuous binding may constitute the cause of most allergic reactions and autoimmune diseases, and is also thought to provide a first line of defense against pathogens (Parnes, 2004). Despite the relevance of promiscuous antibody binding for health and disease, the molecular mechanisms by which antibodies bind to multiple antigens are poorly understood (Kaur and Salunke, 2015; Laffy et al., 2017). The anti-squalene antibody immunoblots presented here suggest that the antibody binds at least to certain acyclic isoprenoids. The anti-squalene antibodies also seem to cross-react with phytane and, to a lesser extent, pristane, at concentrations one order of magnitude higher than squalane. Even though the molar quantities of acyclic diterpanoids tested in Fig. 1 show comparatively less antibody binding, higher concentration of these biomarkers may account for a stronger antibody signal in natural samples, where pristane and phytane are usually dominant. The response of highly concentrated alkanes may also be important given their relative high abundance, along with other hydrocarbons not tested in this study. Additional analyses that are beyond the scope of this study would be necessary to determine the full range of cross-reactivity of this antibody.

Binding affinity of the antibodies towards pristane and phytane is also supported by its binding to their parent hydrocarbon in sediments, phytol (Fig. 1). Strong intensities obtained with phytol may be due to its rigid stereochemistry and double bond that better reflect the structural conformation of squalene. Because of its double bonds, squalene presents a relatively rigid structure, whereas the saturated hydrocarbon structures of squalane, pristane and phytane have more flexible three-dimensional configurations. Comparison of the Tanimoto structure similarity calculated from two-dimensional structure fingerprints of the hydrocarbons (Bajusz et al., 2015) yields a higher similarity score when squalene is compared with phytol (0.59) rather than with squalane or phytane/pristane (0.43; Supplementary Fig. S2A). Non-evident structure similarities between phytol and the cognate antigen observed by Tanimoto score comparisons may be relevant to understanding the antibody cross-reactivity. Three-dimensional

comparison between possible structure configurations of squalene and phytol also shows the conformational similarity, although non-significant, that may illustrate the flexibility of the antibody's binding site to accommodate diverse epitopes (Supplementary Fig. S2B), as previously shown by binding surface analysis of homology models for isolated anti-squalane single-chain variable fragments (Al Qaraghuli et al., 2015). The paratope (the antibody binding site) is therefore likely composed of a hydrophobic binding pocket that can accommodate at least two adjacent isoprenoid subunits in *cis* configuration, as represented by phytol's structure stereochemistry. On the other hand, the antibody binding of saturated forms therefore likely requires folding of the hydrocarbons into a specific three-dimensional configuration. Consequently, the anti-squalene antibody affinity is weaker towards squalane, pristane and phytane, consistent with the results shown in Fig. 1. The relevance of the conformational configuration of the hydrocarbon to supporting binding affinity with the antibody is also highlighted by the high affinity towards phytol, even though it has a hydroxyl group, suggesting that conformational arrangement is more important for antibody binding than the overall hydrophobicity of the hydrocarbon (Supplementary Fig. S2B).

Regardless of its binding promiscuity, the antibody is able to bind biomarkers in complex matrices of hydrocarbons, represented by the crude oil samples analyzed in Fig. 2. Even though the crude oils contain hydrocarbons not tested in this study, the identification of squalane by GC–MS suggests that antibody binding to squalane in crude oil samples is possible. However, the binding intensities observed by immunoblot were not consistent with the concentration of squalane alone. According to GC–MS quantification, the crude oil coat of 320 μg in Fig. 2 only contains about 0.5 nmoles of squalane, which is below the detection limit of the immunoblot for squalane (10–20 nmoles per dot; Fig. 1). High intensities of immunoblotting signals at low squalane concentration compared to the squalane standards analyzed in Fig. 1 suggest that the antibody is also binding to something else, likely other isoprenoids present in the crude oils, such as the diterpanes derived from phytol.

Pristane and phytane are thought to originate after diagenesis of the phytyl group of chlorophylls and therefore are commonly the most abundant isoprenoid biomarkers in petroleum (Peters et al., 2005). Substantial presence of pristane and phytane observed by GC–MS analysis in one of the crude oil samples suggests binding of the anti-squalene antibody to these biomarkers as well, consistent with the concentration-dependent increase of antibody binding to crude oils shown in Fig. 2. Quantities of pristane and phytane in the membrane coatings of the crude oil sample #2 range from 0.03 to 3.7 nmoles/dot, which are also below the detection limits of the immunoblot for pristane and phytane (Fig. 1). It is therefore likely that the antibodies cross-react with other isoprenoids from the crude oil samples, which sum up over 16 nmoles in the most concentrated coat of the crude oil sample #2. On the other hand, we cannot discount the possibility that the antibodies bound to other acyclic or irregularly branched isoprenoids such as botryococcane, crocetane and lycopane, that were not tested in this study and that may be present in the crude oil samples, contributing to the observed signal intensity. Preliminary analysis of lycopene and carotene extracts (Roh et al., 2013) also suggests antibody binding. However, the intense coloration of extract coatings over PVDF membranes precludes their quantification by the chromogenic detection method used (data not shown). Other detection methods, such as chemiluminescence substrates or the incubation with fluorescent-tagged antibodies, used here for geolipid detection in the rock sections, would provide a possible future alternative approach that could increase resolution and detection limits of immunolabeling techniques.

4.2. In situ detection

As part of their efforts to evaluate biomarker syngeneity in Archean rocks, Brocks et al. (2003) noted that the spatial distribution of contaminants in a rock sample should differ from the distribution of syngenetic compounds. For example, some Hamersley and Fortescue Group drill cores contain decreasing concentrations of low-molecular-weight hydrocarbons from the center to the surface of the rock sample, while the concentration of high-molecular-weight compounds increases with distance from the center of the core. Brocks et al. (2003) attribute this heterogeneous spatial distribution to either surficial contamination or migration of the low-molecular-weight compounds during a pressure release brought about by drilling and core extraction (e.g., live oil effect; Brocks, 2011). Certain gross heterogeneities may indeed indicate contamination, however we suggest that given the proper detection tools, the spatial distribution of molecules relative to sedimentary fabrics can provide more detailed information that could be used for the purposes of testing syngeneity and detecting contamination. For example, by analogy to Steno's Principle of Inclusion, molecular fossils included within primary sedimentary fabrics or macerals would suggest that the molecules themselves are syngenetic (Fig. 4A and B). Although preferential incorporation of contaminants into sedimentary organic matter is possible, it is unlikely that contamination, natural or anthropogenic, would deposit molecules only within isolated laminae or macerals. Similarly, spatial associations with secondary features such as fractures or drill core margins would seem to suggest that the molecules of interest were secondarily emplaced (Fig. 4C and D). The homogenous distribution of hydrocarbons within a uniform rock matrix may present a case in which spatial analysis would be unhelpful for establishing the origins of included hydrocarbons.

GC–MS analyses typically require large sample sizes (grams to tens of grams) to produce a detectable signal, which precludes the possibility of spatial analysis by selectively analyzing organic matter from discrete macerals or horizons by conventional techniques. As a result, syngeneity cannot generally be demonstrated by establishing an association between molecules and organo-sedimentary fabrics in the host rock. Time of flight secondary ion mass spectrometry (TOF-SIMS), which allows for the analysis of molecules ablated from a sample, offers one possible solution – although TOF-SIMS analyses are commonly plagued by surface effects and the absence of standardized spectra (Toporski and Steele, 2004).

The immunodetection approach described here is another possibility, yet a more robust antibody characterization would still be required for its application on rocks where biomarker syngeneity is controversial. Variability in the distribution of organic matter in laminated organic rich sediments commonly exists at the sub-millimeter scale; therefore, traditional GC–MS approaches are unsuitable for fine-scale identification of stratigraphic biomarker associations. The detection of squalane within organic-rich laminae in rocks from the Green River Formation (Fig. 3) demonstrates that a monoclonal antibody detection approach may be an effective means of visualizing the distribution of biomarkers or hydrocarbon types within a rock sample at the scale in which they were deposited. The low number of laminae that exhibited binding, as well as the absence of signal from negative control wells, suggests that the antibodies are binding to squalane, and/or structurally similar acyclic isoprenoids, that are concentrated within the partially mineral-bound organic matter. If antibody binding had been observed in many or all laminae, this most likely would have resulted from non-specific binding. The relatively low concentrations of extractable squalane in these rocks, as detected by GC–MS, is consistent with the immunofluorescence results that

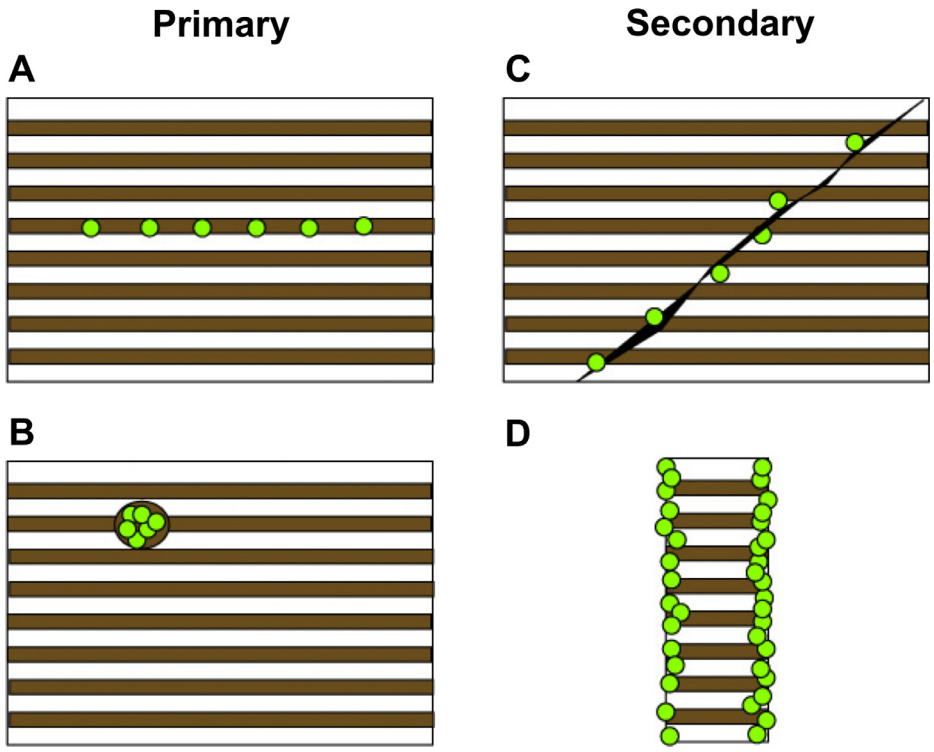

Fig. 4. Schematic representation of hypothetical biomarker syngeneity visualized by immunofluorescence. The localization of biomarkers within primary organo-sedimentary rock fabrics and macerals, such as organic-rich laminae (A) and (micro)fossils (B) is consistent with biomarker syngeneity. Conversely, contamination is suggested by the association of biomarkers with secondary features such as fractures (C), or patterns which are suggestive of diffusion, such as those in illustration D – a cartoon of a core sample contaminated by drilling fluids.

suggest squalane is restricted to only a few discrete macerals. Given the cross-reactivity of these antibodies to concentrated pristane and phytane, they may also have reacted with other isoprenoids, or similar polymer-bound structures within the kerogen matrix. This possibility seems reasonable, given the low concentration of small area of organic matter exposed for binding on each etched slab face.

Ancient rocks that contain organic matter of questionable provenance generally have low concentrations of biomarkers and it is possible that these anti-squalene antibodies would not be sensitive enough to provide an in situ detectable signal of syngenetic compounds at the microscale (Brocks et al., 2003). Antibody binding requires surface exposure of the lipids at concentrations higher than the detection limit of the technique, which may explain the low number of laminae showing a signal. According to Fig. 1, the limit of detection for squalane is 20 nmoles/dot (2.4 nmoles/mm^2), which translates to the microscale as 2.4 fmoles/μm^2 (1 pg/μm^2). Therefore, the visualization of contaminants at the microscale may be limited to above-femtomolar concentrated spots and not to low-abundance lipids homogeneously distributed at the macroscale. Unfortunately, the spatial heterogeneities of potential non-syngenetic biomarkers in controversial samples are far from understood. The future production of other monoclonal antibody lines and their characterization coupled with more sensitive reporters may yet provide important clues regarding biomarker syngeneity in ancient rocks.

5. Conclusions

Antibodies are powerful tools for molecular detection. Targeted antigens for biological applications are typically complex macromolecules, such as proteins. However, numerous studies document the production of antibodies to lipids and membrane-rigidifying lipid compounds such as cholesterol. This study demonstrates, for the first time, that monoclonal antibodies can be used for the in situ detection and visualization of geolipids within partially mineral-bound organic matter. Immunoblotting showed that anti-squalene antibodies bind to squalane and other structurally similar acyclic isoprenoids. Although we cannot discount antibody binding to other compounds, it appears that the binding to other common hydrocarbons, such as the *n*-alkanes and steroids tested in this study, is negligible. In addition, the antibodies were able to bind biomarkers within a complex hydrocarbon matrix represented by the crude oil samples analyzed. Immunofluorescence analysis of squalane-containing, organic-rich mudstones from the Eocene Green River Formation demonstrated that anti-squalene antibodies bind to isolated portions of organic-rich laminae. These findings are significant for organic geochemical studies in that they allow for the association of molecules with organo-sedimentary fabrics, providing critical evidence of syngeneity. Furthermore, paleontological or sedimentological features of the host organic accumulation may provide important clues about the biological source of certain biomarkers. In situ detection could provide evidence of syngeneity, or even show an association between organic-walled microfossils and biomarkers that could be used to establish their phylogenetic affinities.

Our results suggest that certain monoclonal antibodies have the potential to bind to broad classes of hydrocarbons, including some of those preserved in the rock record. Antibodies that bind to lipids are poorly studied relative to protein-specific antibodies. If anti-lipid antibodies can attain the sensitivity and specificity of their anti-protein counterparts, then they may also serve as tools for the in situ detection of specific lipid biomarkers. Because the potential exists for non-specific binding, immunodetection should be used to augment, rather than replace, conventional GC–MS techniques. Future development of this technique could include

the production of antibodies against other biomarkers that could then be used to detect these compounds in sediments, rocks, fossils and modern cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.orggeochem.2018.05.006.

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