Role of Epicellular Molecules in the Selection of Particles by the Blue Mussel, *Mytilus edulis*

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Abstract. This study provides evidence that the suspension-feeding blue mussel, *Mytilus edulis*, uses biochemical cues to recognize its food. We identified lectins in mucus from the gills and labial palps, two pallial organs involved in the feeding process. These compounds were able to agglutinate rabbit and horse erythrocytes (RBC) and several species of marine microalgae representing different families. Additionally, the agglutination of RBC and microalgae was inhibited by several carbohydrates (fetuin, lipopolysaccharide (LPS), and mannose-related residues), suggesting that a suite of lectins may be present in mucus from the gills and labial palps. Results from feeding experiments, using microspheres with tailored surfaces, demonstrated that mussels preferentially ingested microspheres coated with the neoglycoproteins glucosamide-BSA and mannopyranosyl-phenyl-BSA but rejected in pseudofeces microspheres coated with BSA alone. The positive selection for neoglycoprotein-coated microspheres was inhibited when mussels were pre-incubated in seawater containing a solution of the same neoglycoprotein. Two surface properties of the microspheres, charge and wettability, had little effect on the observed selection process. Our results, along with our previous findings for oysters, suggest a new concept for the mechanism of particle selection in bivalves and perhaps other suspension-feeding organisms. Specifically, the selection of particles involves interactions between epiparticulate carbohydrates and lectins in the mucus produced by feeding organs.

Introduction

In near-shore waters, suspension-feeding bivalves are confronted with a wide range of living and nonliving particles. Through several processes, bivalves are able to sort and ingest high-value particles in preference to low-value ones, thus enhancing the nutritive value of ingested material and optimizing energy gain (Allen, 1921; Fox, 1936; Shumway *et al.*, 1985; Defossez and Daguzan, 1996; Pastoureaud *et al.*, 1996; Ward *et al.*, 1997; Ward and Shumway, 2004). The process by which particles are selected is not clear, and several possible mechanisms have been proposed to explain particle sorting, including the recognition of epiparticulate chemical compounds (see details in Ward and Shumway, 2004). Recently, it has been shown that particle processing in *Pecten maximus* (Beninger and Decottignies, 2005) and in *Crassostrea gigas* (Beninger *et al.*, 2008) is directly related to the organic components present on the cell surface of diatoms. Additionally, Pales Espinosa *et al.* (2007) demonstrated that particle selection in the oyster *Crassostrea virginica* is mediated by extracellular metabolites associated with the cell surface of microalgae. Our latest investigations showed that particle sorting in this species results, at least partially, from interactions between carbohydrates associated with microalgal cell surface and lectins present in the mucus covering oyster feeding organs (Pales Espinosa *et al.*, 2009, 2010). Common in many animal species including bivalves (Vasta and Marchalonis, 1982; Suzuki and Mori, 1989; Bulgakov *et al.*, 2004; Tasumi and Vasta, 2007; Takahashi *et al.*, 2008), lectins are a group of non-self recognition proteins that specifically and reversibly bind to sugar residues covering living cells (Sharon and Lis, 2004). Lectins have been shown or suspected to mediate symbiont acquisition in corals (Wood-Charlson *et al.*, 2006), nematodes (Bulgheresi *et al.*, 2006), and the clam *Codakia or-*

Table 1

Microalgal species used in the agglutination and feeding experiments.

Species	Class	Ecological type	Size (μm)
Isochrysis sp.	Prymnesiophyceae	planktonic	5–6
Nitzschia closterium	Diatom (pennate)	benthic/planktonic	17–20
Rhodomonas salina	Cryptophycea	planktonic	15–18
Tetraselmis maculata	Prasinophyceae	planktonic	10–18

bicularis (Gourdine and Smith-Ravin, 2007). Furthermore, the involvement of lectins in the mechanism or mechanisms of prey selection by marine microorganisms was demonstrated by Wootton *et al.* (2007). These authors identified a mannose-binding lectin involved in trophic interactions between the predatory dinoflagellate *Oxyrrhis marina* and *Isochrysis galbana,* its microalgal prey.

Nevertheless, the concept that particle sorting in bivalves is mediated by interactions between microalgal cell-surface carbohydrates and lectins contained in pallial mucus is new and has been demonstrated only in the oyster *C. virginica* (Pales Espinosa *et al.*, 2009, 2010). The aim of the current study was to determine whether lectin involvement in particle selection is common across different bivalve taxa and among species with different gill structures. We chose the blue mussel, *Mytilus edulis*, because this species is capable of qualitative particle selection (Cucci *et al.*, 1985; Ward and Targett, 1989; Bougrier *et al.*, 1997) and possesses a gill structure (filibranch, homorhabdic) different from that of the oyster *C. virginica* (pseudolamellibranch, heterorhabdic). These fundamentally different morphologies have significant functional consequences for particle sorting. For instance, particle selection in mussels is restricted to the labial palps (Ward *et al.*, 1998), whereas in oysters both the ctenidia and labial palps are involved (Ward *et al.*, 1997; Cognie *et al.*, 2003). Therefore, it is possible that the two species use different mechanisms for qualitative selection of their food particles.

To evaluate the involvement of lectin-carbohydrate interactions in particle sorting, we first identified lectins in mucus covering the feeding organs and characterized their carbohydrate specificity. Based on these lectin profiles, we subsequently designed feeding experiments to assess the uptake of microspheres coated with specific carbohydrates and then evaluated the effect of dissolved carbohydrates on particle-sorting efficiency. Surface charge and wettability of the microspheres used in the feeding experiments were also measured. These physicochemical characteristics are widely used to characterize surfaces (Hunter, 1980; Loder and Liss, 1985; Hiemenz, 1986; Razatos *et al.*, 1998) and have been shown to play a role in particle capture in animals that function at low Reynolds numbers (Gerritsen and Porter, 1982; LaBarbera, 1984; Monger and Landry, 1990; Solow and Gallager, 1990; Hernroth *et al.*, 2000). Therefore, these

parameters were considered when we developed conclusions about how lectin-carbohydrate interactions affected particle selection.

Materials and Methods

Organisms

Microalgal strains used in the study (Table 1) were obtained from the Milford Marine Laboratory collection (Connecticut) or were provided by Dr. Nicholas Fisher (Stony Brook University, New York). Microalgae were separately grown in F/2 medium (Guillard, 1982) at 15 °C under a 14-h light:10-h dark cycle. Blue mussels, *Mytilus edulis* (50 to 65 mm shell length), were collected from Long Island Sound, New York and Connecticut. The shells of collected animals were cleaned and encrusting organisms removed. For the feeding experiments, a group of 50 mussels was acclimated to laboratory conditions for 1 week (salinity = 28, measured by means of a refractometer and given as ppt; temperature = 21 °C), during which time they were fed a daily ration (15% dry weight) of DT's Live Marine Phytoplankton (DT's Plankton Farm, Sycamore, IL [Pales Espinosa and Allam, 2006]). Mussels were held in filtered (0.45 μm) seawater for a day prior to being used in the feeding experiments. A second group of mussels was used for the collection of mucus and the agglutination experiments (see below). Eastern oysters, *Crassostrea virginica* (75 to 85 mm shell length, obtained from Frank M. Flower and Sons Oyster Company, Oyster Bay, NY), were used as positive controls for the agglutination assays (Pales Espinosa *et al.*, 2009).

Agglutination of microalgae by pallial-organ mucus

Thirty mussels and oysters were opened and their pallial organs rinsed with filtered artificial seawater (ASW [Ricca Chemical Company, Arlington, TX]: salinity of 30, filtered at 0.22 μm). Mucus covering the gills and labial palps was separately collected using sterile cotton-tipped swabs as previously described (Pales Espinosa *et al.*, 2009). Swabs were then immersed in 60 ml of ASW and stirred for 1 h on a rotating shaker placed in a refrigerator at 4 °C. The resulting fluid (mucous extract) was centrifuged at 400 × g for 15 min (4 °C), filtered (0.22-μm syringe filters) to

Table 2

Effect of 19 carbohydrates on hemagglutination activity of mucous extracts from the gills and labial palps of the mussel Mytilus edulis

		Inhibition of hemagglutination	
Carbohydrates	Concentration	Mucus from gills	Mucus from labial palps
Arabinose·BSA	20 μg · ml^{-1}	+	+
Curdlan	2.5 mg · ml^{-1}	0	0
Dextran	2.5 mg · ml^{-1}	0	0
Fetuin	2.5 mg · ml^{-1}	++	+++
Galactan	2.5 mg · ml^{-1}	+	+
Galactose	36 mg · ml^{-1}	+	+
Galactose-BSA	20 μg · ml^{-1}	++	++
N-Acetyl-galactosamine	44.2 mg · ml^{-1}	++	++
N-Acetyl-glucosamine	44.2 mg · ml^{-1}	+	+
Glucosamide-BSA	20 μg · ml^{-1}	+	++
Glucose	36 mg · ml^{-1}	+	++
Lactose	68 mg · ml^{-1}	++	++
Laminarin	2.5 mg · ml^{-1}	+	+
Lipopolysaccharide	2.5 mg · ml^{-1}	+++	+++
Mannan	2.5 mg · ml^{-1}	+	+
Mannopyranosylphenyl-BSA	20 μg · ml^{-1}	+	++
Mannose	36 mg · ml^{-1}	++	++
Mannose-BSA	20 μg · ml^{-1}	++	++
Sucrose	68 mg · ml^{-1}	0	0

　　Agglutination inhibition was tested with horse red blood cells. + = poor inhibition, ++ = moderate inhibition, +++ = strong inhibition; 0 = no inhibition.

remove debris, and held at 4 °C until used, typically within the following hour. In addition, hemolymph from the adductor muscles of 12 mussels was withdrawn with a syringe equipped with a 22-gauge needle and centrifuged at 300 × *g* for 5 min to remove hemocytes. The resulting supernatant (= plasma), was used in the agglutination assays as a control. A 25-μl aliquot of plasma and mucous extract from gills and labial palps was also analyzed with a Pierce BCA protein assay reagent kit (Pierce, Rockford, IL), as per manufacturer's recommendations, to determine protein concentrations.

　　In parallel, exponentially growing microalgal cultures (4 species, Table 1) were centrifuged at 400 × *g* for 15 min (15 °C). The pelleted cells were washed once, resuspended in ASW, and maintained at 15 °C until used (within the following hour). Agglutination assays were conducted in 96-well microtiter plates by mixing 30 μl of gill or labial-palp mucous extracts from mussels with 30 μl of a suspension containing 5 × 10^6 cell · ml^{-1} of one microalgal species. Control wells contained 30 μl of microalgal cell suspension and 30 μl of ASW (negative controls) or plasma (positive controls). Mucous extracts from oysters were also used as positive controls (Pales Espinosa *et al.*, 2009). Additionally, horse red blood cells (RBC) were also used because these cells are known to be agglutinated by bivalve lectins (Olafsen, 1986; Fisher and Dinuzzo, 1991). Red blood cells (10% suspensions) were obtained from Lampire Biological Laboratory (Pipersville, PA). They were centri-

fuged at 400 × *g* for 5 min (4 °C), washed once in Alsever's solution and twice in ASW diluted with sterile distilled water to a salinity of 9 (DASW), and resuspended in DASW (2% RBC final concentration). Thirty microliters of this suspension was added to wells containing 30 μl of mucous extracts. Each agglutination assay was performed in triplicate wells. After incubation (1 h at room temperature), plates were examined under an inverted microscope and agglutination levels in each well determined using a semiquantitative scale ranging from 0 (no agglutination) to 5 (strong agglutination) (adapted from Allen *et al.*, 1977). Additionally, agglutination titer against RBC, which corresponds to the highest dilution factor that still yields hemagglutination, was determined using 2-fold serial dilutions.

　　To determine the carbohydrate specificity of lectins, hemagglutination-inhibition assays were performed using selected carbohydrates (Table 2; Sigma Aldrich, USA, and V-Labs, Inc., Covington, LA). Carbohydrates were added to mucous extracts from gills and labial palps, and incubated for 1 h at room temperature before RBC were added. Agglutination assays were then performed as described above.

Selection of carbohydrate-coated microspheres

Microsphere preparation. Two types of carboxylated polystyrene microspheres (6-μm diameter, Bangs Laboratories, Inc., Fishers, IN), fluorescent dragon green (excitation/

emission = 480/520 nm) and fluorescent flash red (660/690 nm), were covalently coated with one of several compounds: bovine serum albumin (BSA; control) or one of two neoglycoproteins—glucosamide-BSA (Glu-BSA) or mannopyranosylphenyl-BSA (Mann-BSA). The PolyLink Protein Coupling kit (Bangs Laboratories) was used according to the manufacturer's instructions. The two neoglycoproteins were selected for use in the feeding experiment because preliminary experiments showed that they inhibit the hemagglutination activity of lectins present in mussel mucus (see Results section). About 500 μg of BSA or one of the two neoglycoproteins were separately bound to 10^8 flash-red or dragon-green microspheres.

Feeding experiments. The general design followed our previously described protocols (Pales Espinosa *et al.*, 2009). Equal numbers of microspheres with surface-coated BSA (fluorescent flash red) and neoglycoprotein (fluorescent dragon green; Glu-BSA or Mann-BSA) were suspended in filtered seawater (2×10^5 particles $\cdot$ ml^{-1} total concentration). Mussels were placed in individual trays and delivered 1 liter of the microsphere suspension. Microspheres were kept suspended by gentle stirring. For each mussel, pseudofeces and feces (biodeposits) were separately collected, using a micropipette, 2 h after onset of production. Biodeposits were then homogenized to disperse aggregates, and each type of microsphere was counted using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). The 488-nm argon and the 635-nm red diode lasers were used for excitation, and microspheres were identified using their fluorescence parameters. A minimum of 10^4 events were analyzed, and the proportions of each microsphere type in the diet and in the biodeposits were calculated and compared (see Data analysis). These data allowed us to determine if mussels were preferentially ingesting one type of microsphere over the other.

Sorting inhibition experiments. To further elucidate the importance of lectin-neoglycoprotein interactions in particle selection, mussels were exposed to dissolved neoglycoproteins prior to and during feeding experiments. These treatments were included to determine if dissolved neoglycoproteins would interact with lectins associated with the feeding organs, thus reducing interactions with microsphere-coated neoglycoproteins. First, mussels were placed in individual trays filled with seawater containing 1 mg $\cdot$ l^{-1} of dissolved neoglycoproteins (Glu-BSA or Mann-BSA). After 30 min of incubation, each mussel was delivered a diet made of equal numbers of microspheres coated with either BSA or the neoglycoprotein that was also present in its feeding water (2×10^5 particles $\cdot$ ml^{-1} total concentration). The procedures described above for feeding experiments were then followed. These data allowed us to determine if

particle sorting by mussels could be inhibited by saturating mucus-associated lectins with neoglycoproteins.

Surface properties of microspheres

To examine the influence of surface properties on particle selection, charge and wettability of the microspheres were determined. Microspheres with covalently attached BSA or neoglycoproteins (Glu-BSA or Mann-BSA) were prepared as described above. After being washed twice with Milli-Q water, a sample of each sphere type was incubated in filtered seawater (0.2 μm, salinity of 15, similar to that used in feeding assays) or in a solution of one of the two neoglycoproteins (1 mg $\cdot$ l^{-1} in filtered seawater, salinity of 15). Incubation with seawater or dissolved neoglycoproteins was done to mimic the conditions in the feeding and sorting-inhibition experiments, respectively.

The surface charge of microspheres was estimated on the basis of their electrophoretic mobility in filtered seawater at a salinity of 15 (pH 7.8). The surface charge of a particle in water is difficult to measure directly due to dissolved ions and adsorption of organic matter (Neihof and Loeb, 1974; Hunter and Liss, 1982). Instead, the sum of the electrostatic potentials around the particle, which is a function of the surface charge and termed the zeta potential, is determined (Abramson *et al.*, 1942). For this measurement, microspheres were first incubated in 2-ml microcentrifuge tubes filled with 1 ml of one of the treatment solutions (as described above) for 30 to 60 min. After incubation, each suspension was transferred to a disposable capillary cell and analyzed for zeta potential using a Zetasizer-nano-ZS instrument (Malvern Instruments, Inc.). The accuracy and precision of this instrument is greatest when measurements are made in solutions with an ionic strength less than or equal to a salinity of 15. Standard operating procedures were used with a monomodal setting, a run temperature of 20 °C, and 19 sampling runs per determination. A new disposable capillary cell was used for each replicate determination because the high ionic conditions corroded the cell's electrodes. Three replicate determinations were made for each of the different microsphere suspensions. Replicates were analyzed in an alternating fashion so that all suspensions had replicates that were incubated for 30 to 60 min. Zeta potential was calculated by the instrument using the Smoluchowski-equation setting.

The wettability of microspheres was determined by measuring the contact angle between a pad of the spheres and a drop of water in air (Hiemenz, 1986). Wettability is a function of hydrogen bonding and a rough indication of the surface free energy of the solid. In general, a hydrophilic surface produces a contact angle less than 90°, whereas a hydrophobic surface produces a contact angle of greater than 90° (Christenson, 1992; Good and van Oss, 1992). For this measurement, microspheres were first incubated for 30

min in 15-ml Falcon tubes filled with 5 ml of the appropriate solution. Tubes were then spun and supernatant removed. Ten milliliters of isotonic ammonium formate was added to displace salts (Pales Espinosa *et al.*, 2009), and tubes were sonicated for 15 to 30 s in a bath sonicator to resuspend the microspheres. Suspensions of each sphere type and treatment were passed through individual 3-μm polycarbonate filters (2.5-cm diameter) to form a particle pad that completely covered the filter. Filters were then transferred to glass microscope slides and dried at 70 °C overnight. A 4-μl drop (Della Volpe *et al.*, 2006) of Milli-Q water was placed on each pad and immediately photographed with a digital camera mounted on a horizontal dissecting scope (*ca.* 35× magnification). Contact angles were then calculated from digitized images using Image J software, ver. 1.38x (Abramoff *et al.*, 2004), with the contact angle plugin. Two pads were prepared for each microsphere type and treatment, and a minimum of three replicate determinations were made. Blanks were also prepared by passing several milliliters of filtered seawater through polycarbonate filters and rinsing with ammonium formate. Surface roughness was assumed to be similar for all particle pads.

Data analysis

Data obtained from the feeding experiments were analyzed using goodness-of-fit tests (*G* test). Two series of tests were performed comparing the proportion of each type of microsphere in samples of the diet and biodeposits (pseudofeces, feces) collected from the mussels. The first series of tests ensured that, within each treatment, replicate samples of the diet, pseudofeces, and feces were homogeneous. The second series tested the null hypothesis that, within each treatment, the proportion of each microsphere type in the diet and the proportion of each in the pseudofeces or feces were not different. In addition to the comparison of raw counts, a sorting efficiency (SE) index was calculated in order to examine particle selection (Iglesias *et al.*, 1992). This index was defined as

$$SE = 1 - (P/D),$$

where *P* and *D* represent the proportion of the particle of interest in the pseudofeces and diet, respectively. A positive SE for a given microsphere type indicates that it is preferentially ingested (*i.e.,* lower proportion in the pseudofeces compared to the diet), a negative SE indicates rejection (*i.e.,* higher proportion in the pseudofeces compared to the diet), and zero indicates the absence of selection. After confirming a normal distribution, calculated values of SE obtained for each of the two microsphere types in each treatment were compared to zero using a one-sample Student's *t*-test (two-tailed). The null hypothesis was that the selection efficiencies were equal to zero (*i.e.,* no selection). Two-sample *t*-tests were then used to examine differences in SE among

mussels exposed or not exposed to dissolved neoglycoproteins.

Zeta-potential data for the three microsphere types (surface-coated BSA, Glu-BSA, or Mann-BSA) incubated in filtered seawater or a solution of one of two neoglycoproteins were analyzed using two-way analysis of variance procedures (ANOVA), followed by pairwise comparisons of means (Bonferroni). Sphere type and treatment were used as independent variables. Data were examined for normality and homoscedasticity prior to analysis. Contact angles were analyzed with a similar design, but procedures for circular-scale data were used (Watson-Williams *F*-test; Zar, 1984; Oriana Software, ver. 2.02, RockWare, Inc.). Unless otherwise indicated, all data are given as mean ± standard deviation (SD). For all statistical tests, a significance level of $\alpha = 0.05$ was used.

Results

Agglutination of microalgae by pallial-organ mucus

Protein concentration in plasma (557 ± 34 μg · ml^{-1}), and mucous extracts from gills and labial palps (1180 ± 87 and 335 ± 23 μg · ml^{-1}) were standardized to 300 μg · ml^{-1} by dilution with ASW. Plasma and mucous extracts from *Mytilus edulis* agglutinated horse erythrocytes with comparable intensities (5.0 ± 0.3, 4.0 ± 0.1, and 4.5 ± 0.1, respectively), whereas no agglutination was found in the ASW control (Fig. 1). Both mucous extracts agglutinated three out of the four tested, taxonomically different, microalgal species (Fig. 1). Only *Nitzschia closterium* was poorly agglutinated by mucous extracts from gills (0.04 ± 0.2) and labial palps (0.12 ± 0.05). Agglutination levels ranged from 1.5 ± 0.5 for *Isochrysis* sp. incubated with gill mucous extracts to 5.0 ± 0.1 for *Rhodomonas salina* incubated with mucous extracts from the labial palps. It is noteworthy that the agglutination level was systematically higher for all algal species when tested with mucus collected from labial palps compared to gills. Further analysis using horse RBC demonstrated that the specific activity of lectins contained in labial-palp mucus from *M. edulis* was higher than the specific activity of lectins contained in gill mucus (Table 3).

Results of hemagglutination-inhibition experiments showed that the activity of lectins in mucous extracts from gills and labial palps was not inhibited by curdlan, dextran, or sucrose (Table 2). Hemagglutination activity of lectins was poorly inhibited by the presence of arabinose-BSA, galactan, galactose, *N*-acetylglucosamine, laminarin, and mannan. The highest inhibition of hemagglutination was obtained in the presence of lipopolysaccharide, fetuin, galactose-BSA, *N*-acetylgalactosamine, lactose, mannose, mannose-BSA, and to a lower level, glucosamide-BSA and mannopyranosylphenyl-BSA.
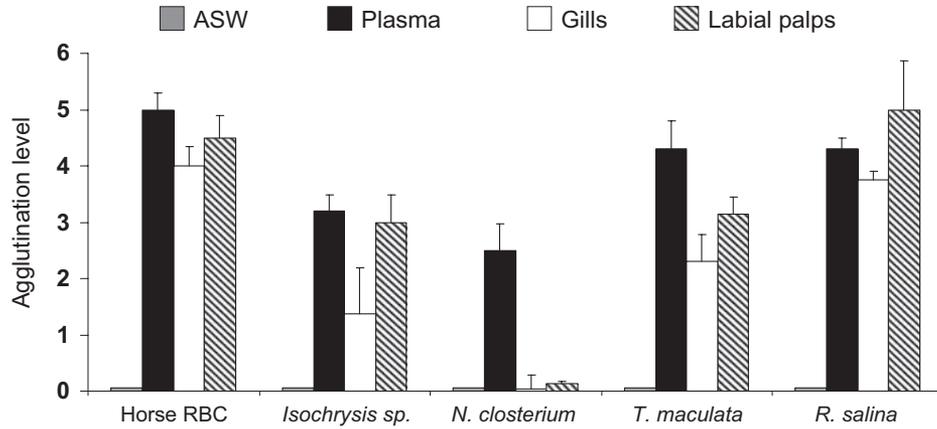
Figure 1. Agglutination activity of plasma and mucous extracts from the gills and labial palps of the mussel *Mytilus edulis*. Agglutination assays were performed using horse red blood cells (RBC) and several microalgal species. Artificial seawater (ASW) was used as the negative control. Data are mean ± SD for 3 replicate measurements.

Selection of carbohydrate-coated microspheres

Two feeding experiments were conducted with mussels given BSA-coated microspheres (control) and microspheres coated with either (1) Glu-BSA or (2) Mann-BSA. In the first experiment, although the targeted proportion of each microsphere was 50% in the diet, an *a posteriori* count revealed a proportion of 39.4% ± 1.6% BSA-coated spheres to 60.6% ± 1.6% Glu-BSA-coated spheres in the suspension. In the second experiment, an *a posteriori* count revealed a proportion of 51.9% ± 1.9% BSA-coated spheres to 48.1% ± 1.9% Mann-BSA-coated spheres in the diet suspension. These proportions remained stable over the course of the experiment, indicating that differential settling of microspheres had not occurred.

In experiment 1, the proportion of BSA-coated microspheres significantly increased ($n = 8$, G test, $P = 0.01$) in the pseudofeces of mussels (47.8% ± 0.7%) compared to the diet, whereas the proportion of the Glu-BSA-coated spheres decreased (52.1% ± 0.7%). The trends in feces were opposite to those of the pseudofeces, with an increase in the proportion of Glu-BSA-coated microspheres (57.4% ± 5.4%) and a decrease in the proportion of BSA-coated spheres (42.6% ± 5.4%). Sorting efficiencies (SE) confirmed the above results. Mussels preferentially ingested Glu-BSA-coated microspheres but rejected BSA-coated spheres (Fig. 2, $n = 8$, t-test, $P <$ 0.001). Results obtained in experiment 2 (*i.e.,* BSA-coated and Mann-BSA-coated microspheres) were similar to those of experiment 1. Calculated values of SE showed that mussels preferentially ingested Mann-BSA-coated microspheres, while they rejected BSA-coated spheres (Fig. 2, $n = 8$, t-test, $P < 0.001$).

Table 3

Specific activity of lectins in mucous extracts from the gills and labial palps of the mussel Mytilus edulis (n = 3 pools of 20 mussels each) and the oyster Crassostrea virginica (n = 2 pools of 20 oysters each)

Species	Pool #	Mucus origin	Total protein mg	Titer *A.U.*	Specific activity *A.U./ml/mg*	Palp/Gill specific activity ratio
M. edulis	1	Gill	1.18	64,000	54,237	1.79
		Palp	0.33	32,000	96,970	
	2	Gill	0.93	32,000	34,411	2.50
		Palp	0.19	16,000	85,879	
	3	Gill	0.84	32,000	38,193	2.11
		Palp	0.20	16,000	80,605	
C. virginica	1	Gill	1.72	16,000	9,302	0.19
		Palp	1.13	2,000	1,770	
	2	Gill	0.98	16,000	16,343	0.30
		Palp	0.81	4,000	4,975	

Agglutination assays were performed using horse red blood cells.

■ BSA-coated microspheres   □ Glu-BSA-coated microspheres
▨ Mann-BSA-coated microspheres
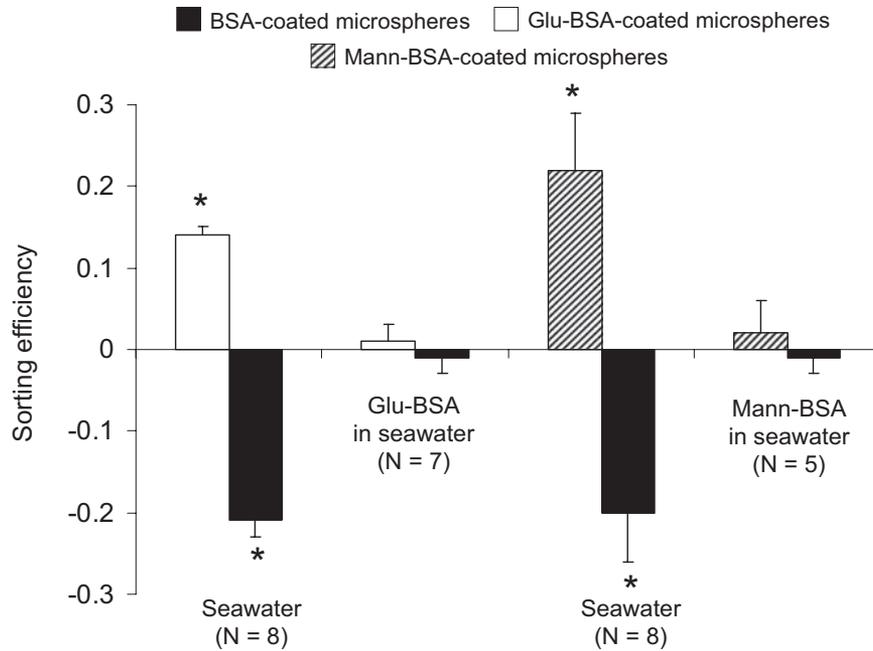
Figure 2. Sorting efficiencies of the mussel *Mytilus edulis* fed a mixture of BSA- (black) and neoglyco-protein-coated microspheres in the absence or presence of the corresponding neoglycoprotein dissolved in seawater (white: Glu-BSA-coated; striped: Mann-BSA-coated). * indicates a sorting efficiency that is significantly different from 0 (one-sample *t*-test, $P < 0.001$). Data are mean ± SD, with number of replicate mussels given in parentheses.

Sorting inhibition experiments

Two sorting inhibition experiments were also conducted by exposing mussels to a mixture of two types of spheres (target proportion for the sphere types was 50/50). In the first, where mussels were exposed to a solution of Glu-BSA, an *a posteriori* count revealed a proportion of 41.1% ± 1.6% BSA-coated spheres to 58.9% ± 1.6% Glu-BSA-coated spheres in the diet suspension. In the second, where mussels were exposed to a solution of Mann-BSA, an *a posteriori* count revealed a proportion of 33.9% ± 2.7% BSA-coated spheres to 66.1% ± 2.7% Mann-BSA-coated spheres in diet. As in the selection experiments, these proportions remained stable over the course of the experiment, indicating that differential settling of microspheres had not occurred.

When mussels were exposed to dissolved Glu-BSA in seawater, the proportion of the two microsphere types in the pseudofeces and feces did not change compared to the diet ($n = 7$, G test, $P > 0.05$). Analysis of sorting efficiencies (SE) confirmed that mussels did not preferentially ingest or reject either microsphere type (Fig. 2, $n = 7$, *t*-test, $P > 0.05$). Similarly, when exposed to dissolved Mann-BSA, the proportion of the two microsphere types in the pseudofeces and feces did not change compared to the diet ($n = 5$, G test, $P > 0.05$). As with the first experiment, no particle selection occurred as SE values were not significantly different from

zero (Fig. 2, $n = 5$, *t*-test, $P > 0.05$). It is noteworthy that the clearance rates of mussels exposed to the control (seawater) and to solutions of the neoglycoproteins were not significantly different (results not shown, *t*-test, $P > 0.05$).

Surface properties of microspheres

Experiments in which glucosamide was used as a treatment produced the following results. There was a significant effect of microsphere type (surface-coated BSA *vs.* Glu-BSA) and treatment (seawater *vs.* solution of Glu-BSA) on zeta potential (Table 4A, $n = 12$, two-way ANOVA, $P < 0.01$). Microsphere type also had a significant effect on contact angle, but treatment did not ($n = 16$, two-way Watson-Williams F, $P < 0.01$ and $P > 0.05$, respectively). Experiments in which mannopyranosylphenyl was used as a treatment produced the following results. Microsphere type (surface-coated BSA *vs.* Mann-BSA) had a significant effect on zeta potential, but treatment (seawater *vs.* solution of Mann-BSA) did not (Table 4B, $n = 12$, two-way ANOVA, $P < 0.01$ and $P > 0.05$, respectively). Microsphere type and treatment also had significant effects on contact angle ($n = 16$, two-way Watson-Williams F, $P < 0.01$). Importantly, in all cases there was a significant difference in zeta potential and contact angle between BSA-coated and neoglycoprotein-coated microspheres, regardless of treatment (Bonferroni and Watson-Williams pairwise comparisons, respec-

Table 4

Surface properties of microspheres (6 μm) with different coatings and treated with seawater or one of two neoglycoprotein solutions

A.	Zeta Potential (mV)		Contact Angle (°)	
Microsphere Type	Seawater	Sol Glu-BSA	Seawater	Sol Glu-BSA
BSA-coated	−8.8 (1.0) a	−6.2 (0.6) b	88.2 (5.0) a	87.6 (3.4) a
Glu-BSA-coated	−4.6 (0.8) b, c	−3.8 (0.6) c	42.6 (5.5) b	49.3 (3.0) b

B.	Zeta Potential (mV)		Contact Angle (°)	
Microsphere Type	Seawater	Sol Mann-BSA	Seawater	Sol Mann-BSA
BSA-coated	−8.8 (1.0) a	−7.8 (0.9) a	88.2 (5.0) a	102.8 (5.3) b
Mann-BSA-coated	−4.6 (0.5) b	−5.0 (0.6) b	46.8 (5.5) c	57.7 (1.8) d

Note: Zeta potentials are presented as means of three replicate measurements ± standard deviation in parentheses. Contact angle data are given as mean vector (μ) of 4 to 5 replicate measurements ± circular standard deviation in parentheses. Within each of the four data sets, means with the same letters are not significantly different at the $\alpha = 0.05$ level (Bonferroni pairwise comparisons).

A. Surface properties of microspheres (6 μm) coated with either bovine serum albumin (BSA) or glucosamide-BSA (Glu-BSA), and treated with either filtered seawater (0.2 μm) or a solution (1 mg · l^{-1}) of glucosamide-BSA (Sol Glu-BSA).

B. Surface properties of microspheres (6 μm) coated with either bovine serum albumin (BSA) or mannopyranosylphenyl-BSA (Mann-BSA), and treated with either filtered seawater (0.2 μm), or a solution (1 mg · l^{-1}) of mannopyranosylphenyl-BSA (Sol Mann-BSA).

tively; Table 4). Contact angles of all microsphere pads were significantly different from those of seawater-treated, blank filters (mean vector = 59.4° ± 3.0° circular SD, $n = 6$), except for microspheres with surface-coated Mann-BSA treated with a solution of Mann-BSA (Watson-Williams F, $P < 0.01$). In this case, the treatment increased the contact angle of the spheres, making it similar to that of the blank. Microscopic analysis of the sphere pads, however, confirmed that microspheres completely covered the underlying filter.

Discussion

Although several studies have previously described the ability of suspension-feeding bivalves to select food particles prior to ingestion and reject undesirable material as pseudofeces (Pastoureaud *et al.*, 1996; Ward *et al.*, 1998; Ward and Shumway, 2004), the mechanisms of this process have remained unclear. Recently, we proposed that particle selection in the oyster *Crassostrea virginica* is at least partially mediated by interactions between lectins contained in mucus covering feeding organs and carbohydrates present at the surface of food particles (Pales Espinosa *et al.*, 2009). The present study adds additional data to support our working hypothesis that carbohydrate-lectin interactions are important for particle recognition in suspension-feeding bivalves. In particular, we have demonstrated that such interactions can mediate particle selection in *Mytilus edulis*, the blue mussel, a bivalve with a different gill architecture and strategy for particle sorting than that of the oyster.

Lectins are a group of carbohydrate-binding proteins that specifically and reversibly bind to sugar molecules covering living cells (Sharon and Lis, 2004). These molecules are well known to play an important role in the recognition of foreign particles and, more specifically, in the defense system of vertebrates and invertebrates. Additionally, lectins are now recognized to be important in diverse processes in marine organisms that involve interactions between microalgae and invertebrates, such as symbiosis (Jimbo *et al.*, 2000; Bulgheresi *et al.*, 2006; Wood-Charlson *et al.*, 2006; Kvennefors *et al.*, 2008) and feeding processes (Wootton *et al.*, 2007; Pales Espinosa *et al.*, 2009). In the current study, we have shown that mucus covering the feeding organs of *M. edulis* contains lectins with diverse activity. Several carbohydrates with different structures were able to partially inhibit agglutination, suggesting the presence of a suite of lectins in mucus both from gills and from labial palps. In species of the Mytilidae, lectins have been identified in extracts from the whole animal (Belogortseva *et al.*, 1998), in hemolymph (Tunkijjanukij *et al.*, 1997; Barracco *et al.*, 1999; Jayaraj *et al.*, 2008), and in association with gametes (Takagi *et al.*, 1994; Springer and Crespi, 2007). Additionally, the development of expressed-sequence-tag libraries has allowed for the identification of several specific lectins from *M. edulis* (Tanguy *et al.*, 2008). Furthermore, the sialic-acid-binding lectin from *Modiolus modiolus,* the horsemussel (Tunkijjanukij *et al.*, 1997), and the β-glucan-binding protein from *Perna viridis,* the green mussel (Jayaraj *et al.*, 2008), are able to agglutinate bacteria and are thought to be involved in the defense mechanism in these species. Lysin-M7, which is thought to be a member of the C-type lectin family (Springer and Crespi, 2007), has been hypothesized to mediate sperm-egg interaction in *Mytilus*

galloprovincialis. Our study is the first to report the presence of lectins in mucus covering the pallial organs of the mussel *M. edulis*. Particle selection has been described in several mussel species (Ward *et al.*, 1998; Ke and Wang, 2002; Velasco and Navarro, 2002; Pales Espinosa *et al.*, 2008) including *M. edulis* (Kiorboe *et al.*, 1980; Cucci *et al.*, 1985; Newell *et al.*, 1989; Ward and Targett, 1989; Bougrier *et al.*, 1997; Defossez and Hawkins, 1997). Our results indicate that the sorting mechanism of *M. edulis* is mediated by recognition molecules on the surfaces of particles. For instance, when mussels were fed microspheres coated with carbohydrates known to inhibit hemagglutination activity (*e.g.,* Glu-BSA or Mann-BSA), a significantly greater number of these spheres were ingested compared to microspheres coated only with BSA, which were rejected in pseudofeces. More interestingly, the pre-incubation of mussels in seawater containing the dissolved neoglycoproteins inhibited selection of microspheres with the same surface-coated carbohydrate. The dissolved carbohydrates probably interacted with lectins contained in the mucus of the gills and labial palps, reducing subsequent interactions with carbohydrates bound to the microspheres and resulting in loss of particle-sorting capabilities. These results suggest that particle selection in mussels relies on a recognition mechanism involving lectins that interact with glucose and mannose residues.

Microspheres coated with the neoglycoproteins had significantly lower zeta potentials and contact angles than the same microspheres coated with BSA alone. Although treating these microspheres with a solution of neoglycoprotein significantly reduced zeta potential (*i.e.,* BSA- and Glu-BSA-coated microspheres treated with a solution of Glu-BSA), or significantly increased contact angle (*i.e.,* BSA- and Mann-BSA-coated microsphere treated with a solution of Mann-BSA), the significant differences between the two microsphere types remained (Table 4). These data suggest that surface charge and wettability had little effect on particle selection in our experiments. Mussels preferentially ingested microspheres coated with neoglycoproteins when exposed only to seawater, but demonstrated no selection when exposed to a solution of neoglycoprotein in seawater, even though under both treatments the surface characteristics of the two microsphere types were different.

Lectin-specific activity in mussels was noticeably higher in mucous extracts from the labial palps than in extracts from the gill (Table 3). The opposite trend was observed in the oyster *Crassostrea virginica*, which demonstrated higher specific activities in mucous extracts from the gill (Table 3). This dissimilarity may reflect differences in the functional morphology of the feeding organs and locus of particle selection. *Mytilus edulis* possesses a filibranch, homorhabdic gill composed of a single filament type, whereas *C. virginica* possesses a pseudolamellibranch, heterorhabdic gill composed of two different filament types. Results of

previous studies indicate that the heterorhabdic gill of several oyster species (*i.e., Crassostrea virginica* and *C. gigas*) is capable of particle selection. In mussels (*i.e., M. edulis, M. trossulus*), however, the homorhabdic gills function little in particle sorting, and the labial palps are the main site of selection (Ward *et al.*, 1997, 1998; Levinton *et al.*, 2002). Therefore, the importance of each organ in the selection process of mussels and oysters matches well with their specific lectin activity. It is possible that the degree of involvement of each organ in particle selection is reflected in its lectin content, but this assessment remains highly speculative without further investigation.

Results obtained here for the blue mussel complement our prior work for the eastern oyster (Pales Espinosa *et al.*, 2009), and provide additional evidence that particle sorting in suspension-feeding bivalves involves interactions between epiparticulate/cellular carbohydrates and lectins present in mucus covering the feeding organs. The identification and isolation of mucosal lectins would allow, at a molecular level, for a precise exploration of the mode of operation of these sugar-binding proteins in particle selection.

Acknowledgments

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