Impact of Polymer Surface Affinity of Novel Antifouling Agents

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Abstract: In a previous study we found two agents, the α_2-agonist medetomidine ((±)-4-[1-(2,3-dimethylphenyl)ethyl]-1*H*-imidazole) and the α_2-agonist clonidine (2-(2,6-dichloroanilino)-2-imidazoline), that specifically and efficiently impede settlement of the barnacle *Balanus improvisus*, one of the most serious biofouling organisms in Swedish waters. Medetomidine, but not clonidine, is known to adsorb to solid polystyrene (PS) surfaces in the presence of salt, a feature that is of particular interest in attempts to develop an efficient antifouling surface.We show that medetomidine, but not clonidine, has a significant ability to adsorb to untreated (hydrophobic) PS in two different incubation media: filtered seawater (FSW) and deionized water (mQ). At negatively charged (hydrophilic) PS, medetomidine displays a strong interaction with the surface in both incubation media. At the hydrophilic PS, clonidine also displays a significant interaction with the surface when incubated in mQ and a weaker, but not significant, interaction when incubated in FSW. By studying the effects of time, incubation media, and pH on the adsorption of medetomidine and clonidine, we suggest that medetomidine is associated to hydrophobic PS by means of hydrophobic interactions, while the adsorption of medetomidine and clonidine to hydrophilic PS contains elements of electrostatic interaction. Using time-of-flight secondary ion mass spectroscopy (TOF-SIMS) we detected only weak signals from medetomidine on the hydrophobic PS surfaces, while strong medetomidine signals were observed on hydrophilic PS. This suggests that the adsorbed medetomidine, to a greater extent, desorbed from the hydrophobic rather than from the hydrophilic PS surfaces during exposure to vacuum. The strong surface affinity of medetomidine on both types of surfaces and the preserved antifouling activity are valuable features in designing a marine coating. © 2004 Wiley Periodicals, Inc.

Keywords: α_2 agonists; fouling; barnacle; surface affinity; cyprid larvae; TOF-SIMS

INTRODUCTION

Man-made surfaces immersed in the sea will rapidly be covered by biological macromolecules such as carbohydrates and proteins, and within weeks, the surface will be progressively overgrown by bacteria, algae, and invertebrates in a process called biofouling. Biofouling on the hulls of boats and ships causes great economic losses within the shipping industry through increased friction, fuel consumption, and material fatigue. Organisms with calcareous hard body armors, such as barnacles and polychaete tubeworms, are particularly serious in terms of friction and resistance to cleaning (Berntsson, 2001; Harder and Qian, 2000). These organisms readily colonize any surface submerged in the sea and form dense populations. The standard method to combat biofouling is to include antifouling agents in the paint formulation. However, several of the toxic substances now used for antifouling purposes are facing bans because of nontarget effects in the marine environment (reviewed by Fent, 1996), and the search for new, nontoxic antifouling methods is intense.

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The barnacle *Balanus improvisus*, which is the target organism of this study, has several pelagic naupliar stages before molting into the non-feeding settlement stage, the cyprid larva. When barnacle cyprid larvae encounter a potential settling site, they exhibit a specific surface-bound behavior, during which they explore the surface by means of their antennae (Berntsson et al., 2000; Crisp, 1974). During the exploration of the substratum, the cyprid larva is able to perceive and respond to a diverse range of external signals. Chemical signals are possibly perceived by several aesthetasc sensilla, which are believed to be located on the lattice organs situated dorsally on the larvae (Hoeg et al., 1998) and also on the antennular segments (Clare and Nott, 1994; Nott and Foster, 1969). The gregarious settlement in many balanomorph barnacles, like *B. improvisus*, is believed to be a result of chemical signals associated with the surface and derived both from adult conspecifics and from conspecific larvae (Clare et al., 1994; Matsamura et al., 1998; Yule and Walker, 1985). Hence, cyprid larvae are well adapted for receiving signals bound to or associated with the surface. A surface-active compound in an antifouling paint is thus likely to have a greater impact on the settlement of barnacle larvae than a compound leaking out of the paint into the water since surface activity will increase the concentration close to the surface. Compounds with high surface affinities are consequently expected to improve the performance of antifouling paints with regard to barnacle colonization. We have previously described the ability of the two structurally similar substances, medetomidine and clonidine, classified in vertebrate pharmacology as α_2-agonists, to inhibit settlement of barnacle larvae of *B. improvisus* in concentrations ranging from 250 pg mL^{-1} to 2.5 ng mL^{-1} when present in solution (Dahlström et al., 2000). Furthermore, these substances show large EC_{50}:LC_{50} ratios (in the 10^5 range), implying low toxicity to the larvae. We also found that medetomidine, but not clonidine, displayed an obvious tendency to accumulate at hydrophilic and hydrophobic polystyrene (PS) surfaces, where it was able to exert its settlement-inhibiting action (Dahlström et al., 2000).

It is an urgent bioengineering challenge to develop a coating system that permits slow and controlled release of low-toxic, bioactive, antifouling substances. An organic antifouling substance, such as medetomidine, is likely to interact with several components in the paint film, and interaction with the binder can be assumed to be of particular importance since the binder is the dominating organic component in a paint film. The binder in a marine paint formulation is typically a hydrophobic polymer that may contain negatively charged carboxylate groups.

The main objective of the present study is to characterize the effect of the antifouling compounds medetomidine and clonidine at polymer surfaces. Specifically, we attempted to adsorb and desorb medetomidine and clonidine to hydrophilic and hydrophobic PS surfaces under various conditions to characterize the type of surface interactions involved. The cyprid settlement assay was used to ensure that the biological activity of the adsorbed substances was preserved, which can be seen as an indirect assessment of the presence of the substance at the surface. Furthermore, we used time-of-flight-secondary ion mass spectroscopy (TOF-SIMS) to chemically detect the adsorbed compounds.

MATERIALS AND METHODS

Larval Rearing and Cyprid Settlement Assays

Nauplius larvae of the barnacle *B. improvisus* were collected and reared from the brood stock in the culture at Tjärnö Marine Biological Laboratory to yield competent cyprid larvae, as described earlier (Berntsson et al., 2000). All larvae were used on their first or second day of molting. The settlement assays were performed using Petri dishes (Ø 48 mm, Nunc) of hydrophilized polystyrene (PS) (advancing contact angle with mQ water 81.1° ± 1.6°) or untreated PS (advancing contact angle with mQ water 51.4° ± 0.6°). These surfaces have previously been characterized using X-ray photoelectron spectroscopy (XPS), establishing a 12% oxygen content on the hydrophilic PS dishes, while on the hydrophobic PS surfaces no oxygen was detected (Dahlström et al., 2000). The seawater used was collected via pipes from the Koster fjord (depth, 50 m; salinity, 32 ± 1‰) and then filtered through a 0.2-μm filter. Settlement assays were performed under static conditions (Dahlström et al., 2000; Rittschof et al., 1984, 1992), and the experiments were maintained for 6–8 days, after which the dishes were viewed under a dissecting microscope to check for attached and metamorphosed individuals and for living cyprids. The chemicals used in these experiments were obtained from Sigma Aldrich (St. Louis, MO), except for medetomidine, which was generously provided by Orion Pharma Oy (Åbo, Finland). The hydrochloride salts of medetomidine and clonidine (Fig. 1A,B) were dissolved in filtered seawater (FSW) and diluted to give the desired concentrations.

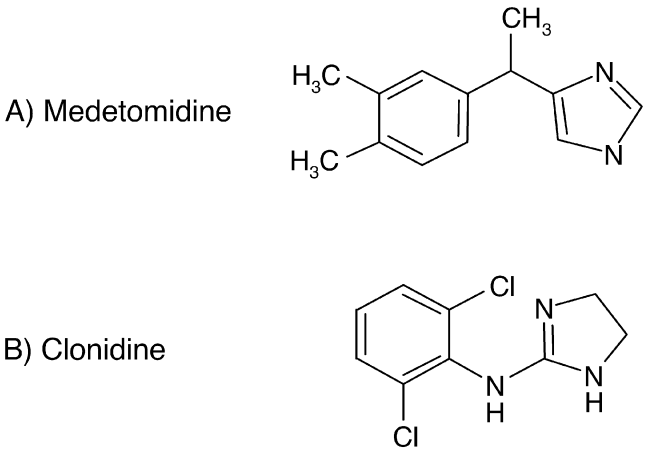

Figure 1. Chemical configurations of (**A**) medetomidine and (**B**) clonidine.

Time-Dependence of Surface Accumulation

A series of experiments were carried out to quantify the response of some important factors involved in determining the surface accumulation of medetomidine. The mass transported from the bulk solution to the solid surface is expected to depend on incubation time and incubation concentration. Different combinations of time and concentration were tested using both hydrophilic PS and hydrophobic PS. Dishes of hydrophilic and hydrophobic PS were filled (20 mL) with 100 ng mL^{-1} or 1 μg mL^{-1} of medetomidine in FSW and were allowed to stand for 4, 8, 16, and 32 min to determine the time needed for medetomidine to accumulate at the surface in sufficient amounts to obtain maximum settlement inhibitory activity. After incubation, the solution was poured off and each dish was washed 4 times with FSW. The dishes were then allowed to equilibrate for 2 min with FSW. This water was poured off, 8 mL of FSW was added together with 20–30 cyprids in each dish, and the experiment was maintained for 6–8 days. Dishes of hydrophobic and hydrophilic PS with FSW (8 mL) served as controls. Each treatment in either type of dish was replicated 3 times, and the experiment was repeated 3 times.

Effect of pH on Surface Accumulation

Medetomidine and clonidine consist of a 1,3-diazacyclopentadiene and a 1,3-diazacyclopentene ring, respectively, connected to a disubstituted benzene ring via a carbon and a nitrogen atom, respectively (Fig. 1A,B). The pK_a value of medetomidine is 7.1, and that of clonidine is around 9; thus, medetomidine can be regarded as partially charged (~10%) and clonidine to be fully charged (~90%) at the pH of seawater (7.9). Two different incubation media were used in the experiments to determine the effect of pH on the surface interaction of clonidine and medetomidine. FSW was used as incubation medium since both clonidine and medetomidine have a potential use as additives in marine coatings, and mQ was used to establish the importance of electrostatic interactions.

In the first experiment, dishes of hydrophilic and hydrophobic PS were incubated with FSW or mQ water containing 1 μg mL^{-1} of medetomidine or clonidine. The pH of the FSW was 7.9 ± 0.1 (normal pH for seawater), 5.6 ± 0.1 or 3.6 ± 0.1, respectively. The mQ water was made basic with 0.5 *M* NaOH (pH 7.9). The FSW and the mQ water were made acidic with 1.2 *M* HCl, and the pH was determined with a pH meter (Hanna Instruments, Woonsocket, RI). The dishes were incubated for 3 h with medetomidine or clonidine. After the incubation, the dishes were rinsed and equilibrated as described above and FSW (8 mL) was added together with 20–30 cyprids. Dishes of hydrophobic and hydrophilic PS, which had been treated in the manner described above except the addition of medetomidine or clonidine were filled with FSW (8 mL) and served as controls. The experiment was maintained for 6–8 days, after which the dishes were viewed under a dissecting microscope and the attached and metamorphosed cyprids were counted. The treatments were replicated 4 times, and each experiment was performed 3 times.

In a second experiment, the rinsing water was made acidic to establish whether the surface bound medetomidine could be desorbed from the surface. Dishes of hydrophilic and hydrophobic PS were incubated with 1 μg mL^{-1} of medetomidine in FSW (pH 7.9) for 2 h, after which they were rinsed 4 times and allowed to equilibrate for 2 min with FSW of pH 7.9, 5.0, 4.0, or 3.0. The rinsing water was made acidic, and the pH was determined in the same manner as described above. FSW (8 mL) was added to each dish together with 20–30 cyprids, and the experiment was maintained for 6–8 days. Dishes of hydrophobic and hydrophilic PS, which had been treated in the manner described above except the addition of medetomidine, were filled with FSW (8 mL) and served as controls. The treatments were replicated 3 times, and each experiment was performed twice.

Analysis of Surface-Bound Medetomidine Using Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS)

Hydrophobic and hydrophilic polystyrene samples were incubated in medetomidine solutions (1 μg mL^{-1}, 30 min at RT) and then analyzed with time-of-flight secondary ion mass spectrometry (TOF-SIMS). Non-incubated samples of the corresponding PS materials were also analyzed for comparison. Positive and negative secondary ion mass spectra were acquired from at least two areas of 500 × 500 μm on each sample; 25-keV Ga^+ ions were used as the primary ions at a beam current of 0.5 pA. The spectra were acquired under static SIMS conditions, i.e., the primary ion dose was kept below 1×10^{13} ions cm^{-2} in order to avoid contributions from ion beam damaged areas in the spectra.

Statistical Methods

Results are generally reported as means ± standard error. Effects of the studied substances on cyprid settlement were tested using one- and two-factor analysis of variance (ANOVA) with all tested treatments as fixed factors (Winer et al., 1991). In all tests an α of 0.05 was used.

RESULTS AND DISCUSSION

To make an in-depth investigation of the factors involved in the surface binding of medetomidine and clonidine (Fig. 1A,B), we used two types of surfaces: one polar and one nonpolar. Previous work has shown that clonidine, in solution, displays approximately the same efficacy in settlement inhibition as medetomidine but appears to lack sufficient surface affinity (Dahlström et al., 2000). The approaches used to determine the mechanisms involved in

the surface interaction of the antifouling substances were to study how time and concentration, as well as pH and type of incubation medium, affected the ability of the substances to impede cyprid settlement. In addition to the biological assay, using cyprid settlement as an indicator of the presence of the substance at the surface, we used the surface sensitive analytical method TOF-SIMS.

Figure 2 shows the cyprid settlement inhibition effect of medetomidine at different times and concentrations. We found that in the hydrophobic dishes incubated with 100 ng mL^{-1} or 1 µg mL^{-1} of medetomidine settlement of cyprid larvae was inhibited in a time-dependent manner (2-factor ANOVA, $F_{4,20} = 6.7$, $P < 0.05$) (Fig. 2A). This time dependence occurred regardless of the concentration used, and hence, there was no significant interaction between time and concentration ($F_{4,20} = 0.2$, $P > 0.05$). In the hydrophilic dishes, settlement was completely inhibited for both concentrations already at the incubation time of 4 min (two-factor ANOVA, $F_{4,20} = 79$, $P < 0.05$) (Fig. 2B). The experiments were conducted three times with the same results. We suggest that the much more pronounced effect obtained in the hydrophilic dishes is an effect of stronger affinity due to electrostatic interactions between medetomidine and this surface. Medetomidine, which is a weak base, will be partially positively charged at the pH used (7.9), thus binding can take place through ionic interactions with negatively charged carboxylate groups present at the surface of the hydrophilic dish (Dahlström et al., 2000). Hydrophobic interactions are also likely to contribute to the binding to the hydrophilic dish. In the binding of medetomidine to the surface of the hydrophobic dish, no Coulombic interaction forces are involved. The interaction must be due to hydrophobic interactions and possibly π–π interactions, primarily between the diazacyclopentadiene ring as electron-rich π component and surface benzene rings as electron-poor π components. Also, π–cation interactions involving the partially protonated amino group of medetomidine as the cation component and surface benzene rings as the π components may play a role. We conclude by this experiment that the electrostatic binding is the most important means of the molecule to interact with the solid surface; however, elements of hydrophobic interactions are also contributing to the surface affinity of medetomidine.

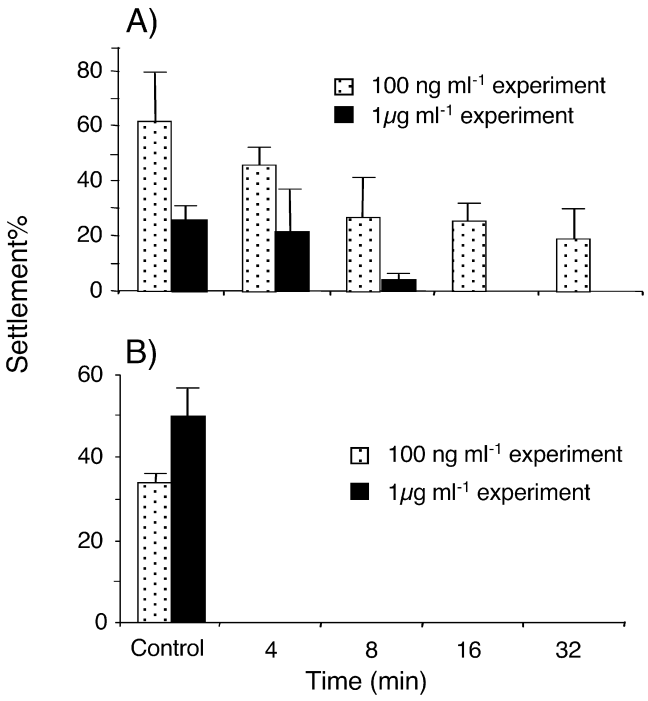

Figure 2. Time-dependence of the surface accumulation of two different concentrations of medetomidine: (**A**) hydrophobic surfaces; (**B**) hydrophilic surfaces. The response variables of percentage settled are shown as means ± SE ($n = 3$). (Note the difference in scale between the two.)

When incubating medetomidine and clonidine in mQ at three different pH values, we found that medetomidine significantly interacted with both types of surfaces in all pH treatments tested. Thus, regardless of incubation pH, the settlement of cyprid larvae was completely inhibited and no significant interaction between the factors treatment (control vs. medetomidine), and pH was obtained at neither the hydrophilic (ANOVA, $F_{2,18} = 0.42$, $P > 0.05$) (Fig. 3A) nor the hydrophobic (ANOVA, $F_{2,18} = 0.35$, $P > 0.05$) (Fig 3B) surfaces. The results from the incubation of clonidine in mQ at different pH displayed the same pattern in the hydrophilic dishes (ANOVA, $F_{2,18} = 1.56$, $P > 0.05$) (Fig. 3A). In all tests, the factor treatment was significant. However, clonidine was still unable to interact with the hydrophobic surfaces when incubated in mQ in all the different pH treatments, and thus the settlement in these dishes equaled that in the control dishes (Fig. 3B).

The pH strongly affected the ability of medetomidine, but not of clonidine, to interact with the hydrophobic surfaces when incubated in FSW. Our results show that medetomidine completely inhibited settlement on hydrophobic surfaces at pH 7.9 and 5.6 (Fig. 3C). However, settlement was not inhibited at pH 3.6 (Fig. 3C). These effects are detected as a significant interaction among the factors pH and treatment (control vs. medetomidine) (two-factor ANOVA, $F_{2,18} = 54.2$, $P < 0.05$). The major contribution to this interaction is the high settlement in the dishes incubated with medetomidine in FSW at pH 3.6. On the hydrophilic surfaces, medetomidine inhibited settlement at all three incubation pH values (Fig. 3D). Thus, it seems that the pH did not affect the ability of medetomidine to adsorb to the surface (two-factor ANOVA, $F_{2,12} = 1.53$, $P > 0.05$). In the dishes incubated with clonidine, the effect of pH was small both on the hydrophilic and on the hydrophobic surfaces (Fig. 3C,D).

In these experiments, one striking result is the inability of medetomidine to interact with the hydrophobic surface when incubated in FSW at pH 3.6. This inability cannot solely be explained in light of intermolecular repulsion, since the molecule is fully charged also at pH 5.6. Furthermore, in the absence of salts, medetomidine is able to interact with the hydrophobic surface regardless of pH.

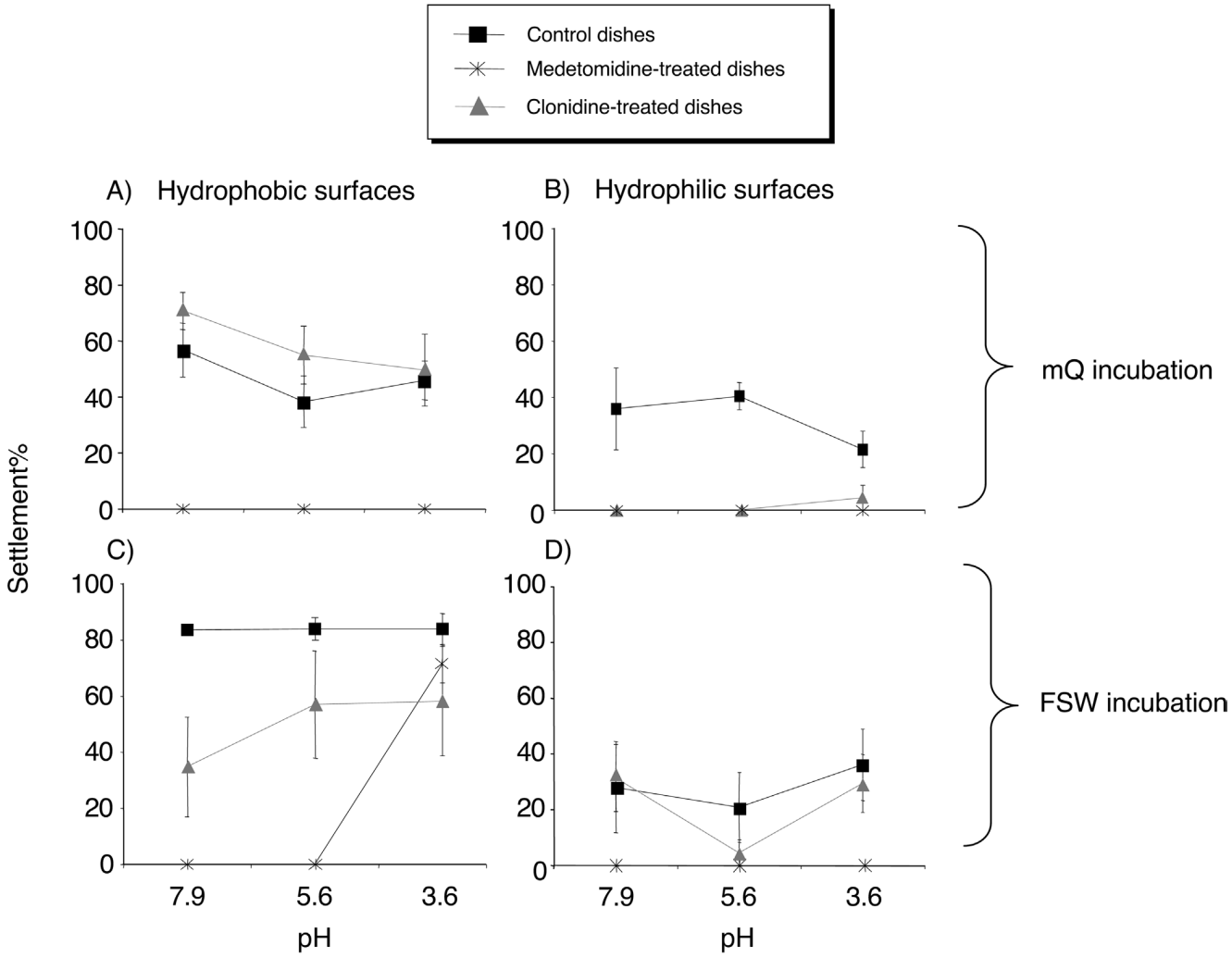

Figure 3. pH dependence of the surface accumulation of medetomidine and clonidine. (**A**) Hydrophobic surfaces, incubation in mQ; (**B**) hydrophilic surfaces, incubation in mQ; (**C**) hydrophobic surfaces, incubation in FSW; (**D**) hydrophilic surfaces, incubation in FSW. The response variable is percentage settled in control dishes, medetomidine-treated dishes, and clonidine-treated dishes shown as means ± SE (n = 4).

We therefore suggest that, when using FSW as incubation medium, the positive charge of medetomidine at pH 3.6 in combination with the high ionic strength of the FSW alters the surface binding behavior to the hydrophobic surfaces. It may be speculated that the presence of salts may increase self-association, thereby reducing interface accumulation. We have made analogous observations concerning the adsorption of medetomidine at different pH values and in the absence and presence (artificial seawater) of salt in experiments using ellipsometry, an optical surface-sensitive method whereby the concentration of medetomidine at the surface can be directly quantified (Arnebrant et al., unpublished). However, we are not yet able to account for the inability of medetomidine to interact with the hydrophobic surface at pH 3.6. Clonidine, on the other hand, seems unable to sufficiently interact with the hydrophobic surfaces, regardless of pH and incubation media. Thus, we propose that the interaction of medetomidine to hydrophobic surfaces contains substantial elements of hydrophobic interactions.

The association of medetomidine and clonidine molecules to the hydrophilic surfaces in the absence of salts is probably mediated by electrostatic interactions to negatively charged carboxylate groups. However, when the pH is lowered and thus, the solubility of the molecules is increased while the surface is also rendered noncharged, the molecules are still able to interact with the surface. It is likely, though, that the amount of substance associated to the surface decreases with lowered pH as a consequence of intermolecular repulsion, a feature that is not detectable by the cyprid larvae settlement assay.

The results of the experiments where the dishes were incubated at pH 7.9 but washed with FSW at low pH (pH 5.0, 4.0, and 3.0) show that desorbtion of the medetomidine molecule is not possible by employing these washing procedures, regardless of surface and pH. In these experiments, the pH of the washing water was not able to affect the medetomidine already associated to the surface in any of the dishes (one-factor ANOVA, $F_{7,16} = 310.7$, $P < 0.05$) (Fig. 4A,B).

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