Antifouling Activity of synthetic polymer gels against cyprids of the barnacle (balanus amphitrite) in vitro

by Ananda Putra

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Antifouling activity of synthetic polymer gels against cyprids of the barnacle (*Balanus amphitrite*) *in vitro*

T. Murosaki[a], T. Noguchi[a], A. Kakugo[a], A. Putra[a], T. Kurokawa[a], H. Furukawa[a], Y. Osada[a], J.P. Gong[a]*, Y. Nogata[b], K. Matsumura[c], E. Yoshimura[d] and N. Fusetani[e]

[a]*Department of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan;* [b]*Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, Abiko 270-1194, Japan;* [c]*Division of Protein Folds Research, International Graduate School of Arts and Sciences, Yokohama City University, Yokohama 230-0045, Japan;* [d]*CERES Inc., Abiko 270-1166, Japan;* [e]*Faculty of Fisheries Sciences, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*

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Barnacle (*Balanus amphitrite*) settlement on synthetic hydrogels with various chemical structures was tested in laboratory assays. The results demonstrated that cyprids settle less or not at all on hydrogels and PDMS elastomer compared with the polystyrene control. The low settlement on gels is most likely due to the 'easy release' of initially attached cyprids from the gel surfaces. This low adhesion of cyprids is independent of surface hydrophilicity or hydrophobicity, and of surface charge. The results also revealed that hydrogels can be categorized into two groups. One group showed an extremely strong antifouling (AF) performance that was independent of the elasticity (E) or swelling degree (q) of the gels. The second group showed relatively less strong AF performance that was E- or q-dependent. In the latter case, E, rather than the q, may be the more important factor for cyprid settlement.

Keywords: polymer; gel; cyprid; barnacle; biofouling; antifouling

Introduction

Marine fouling by macrofoulers is a serious economic problem worldwide. Barnacles are one of the macrofoulers that attach densely to various submerged surfaces, such as fishnets, ships' hulls, and power plant cooling water intake channels. To inhibit fouling by macrofoulers, several antifouling (AF) compounds have been developed. Tributyltin (TBT) is the most popular of these and exhibits high AF performance. However, TBT was banned for use worldwide in September 2008 due to its high endocrine disruption effect. Development of an alternative AF system is urgently necessary to protect submerged surfaces, because currently there is no good replacement for TBT.

Several environmentally benign AF approaches against barnacles have been reported, such as surface coating with natural AF compounds from marine sponges (Fusetani et al. 1996), marine bryozoans (Kon-ya et al. 1994), and whip corals (Rittschof et al. 1985). Another example is a polydimethylsiloxane (PDMS) silicone elastomer coating that has a low surface energy of 20–30 mN m^{-1} (Brady et al. 1987). These AF approaches are based on solid materials, and less attention has been paid to soft, wet materials, such as hydrogels.

It is apparent that certain soft, wet surfaces, such as external layers of dolphins, porpoises, and killer whales in the marine environment, are not fouled by macrofoulers (Baier et al. 1985). A similar AF performance against microfoulers is found in human intraoral mucosa in its saline environment (Glantz et al. 1991). Many years ago, Hempel Marine Paints of Denmark introduced a complete hydrogel as an AF coating based on Hydron™, but it failed in the field. It has been reported that barnacle settlement in the laboratory is inhibited on hydrogels originating from natural resources, such as agarose, alginate, and chitosan, and on photo-crosslinked poly (vinyl alcohol substituted with photo-sensitive stilbazolium groups) (PVA-SbQ) gel (Rasmussen et al. 2002). However, only a few gels have been tested in the laboratory and the AF mechanism of gels against barnacles is poorly understood.

Hydrogels are soft and wet materials, as is generally known. Normally, their elastic modulus lies in a range of 1 kPa–1 MPa and their water content is as high as 50–99.9% of their total weight. They also have extremely low surface frictional forces against themselves or solid substrata (Gong et al. 1997, 1999; Gong 2006). Thus, the surface properties of gels clearly

*Corresponding author. Email: gong@mail.sci.hokudai.ac.jp

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differ from solid materials. Recently, the authors also reported that some hydrogels exhibit AF performance against algae (Katsuyama et al. 2002).

In this article, the settlement behavior of barnacle cyprids (*Balanus amphitrite*) on various synthetic polymer gels was systematically studied in the laboratory, focusing on the effects of polymeric electrical and mechanical properties (elastic modulus) on settlement. There are three reasons for using synthetic polymer gels in a settlement test. Firstly, the chemical and physical properties of the gels can be easily modulated by selecting monomer species; secondly, industrially popular synthetic polymers for making gels can be inexpensively obtained, which would enable mass production in future applications; and thirdly, synthetic polymer gels generally have low biodegradability, which ensures long-term durability in the marine environment. The chemical structures of the monomers, polymers, crosslinker, and initiator that were used in this study are shown in Figure 1. As a soft material reference to the hydrophilic hydrogels, a hydrophobic PDMS silicone elastomer was also used in the study.

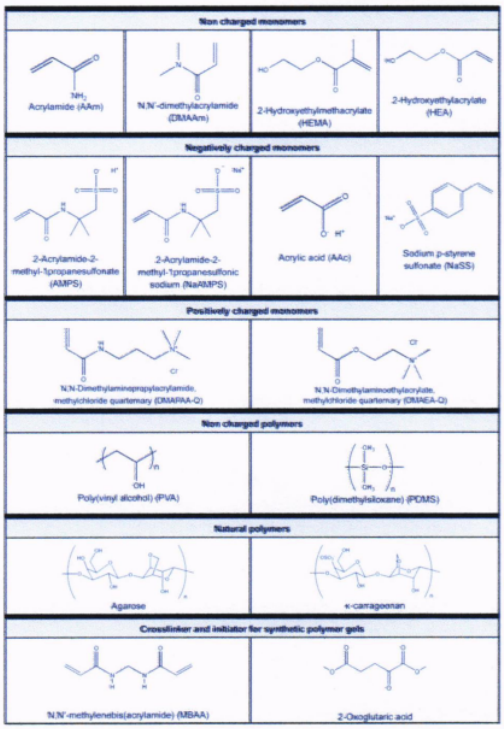

Figure 1. The chemical structures of the monomers, polymers, crosslinker, and initiator used for synthesizing gels.

The settlement test was performed using four types of synthetic polymer gels to investigate the key parameters affecting their AF properties. The first type was chemically crosslinked hydrogels with three different types of charge: (1) non-charged hydrogels, such as poly(acrylamide) (PAAm), poly(*N*,*N*′-dimethylacrylamide) (PDMAAm), poly(2-hydroxyethylmethacrylate) (PHEMA), and poly(2-hydroxyethylacrylate) (PHEA) gels; (2) positively charged hydrogels, such as poly (*N*, *N*-dimethylaminopropylacrylamide, methylchloride quarternary) (PDMAPAA-Q) and poly(*N*,*N*-dimethylaminoethylacrylate, methylchloride quarternary) (PDMAEA-Q) gels; and (3) negatively charged hydrogels, such as poly(sodium *p*-styrene sulfonate) (PNaSS), poly(2-acrylamide-2-methyl-1-propanesulfonate), and poly(2-acrylamide-2-methyl-1-propanesulfonic sodium) (PNaAMPS) gels. The second type was a physically crosslinked non-charged synthetic polymer of poly(vinyl alcohol) (PVA) gel with high tensile strength and high moldability, which was employed for comparison with the chemically crosslinked gels. The third was double network (DN) gels consisting of two interpenetrating polymer networks (Gong et al. 2003) having high mechanical strength, such as PAMPS and PAAm (PAMPS/PAAm DN), and poly(acrylic acid) (PAAc) and PAAm (PAAc/PAAm DN) gels. The fourth type was a chemically crosslinked elastomer poly(dimethylsiloxane) (PDMS), which is a soft but hydrophobic material. Polystyrene (PS), which is a hard and hydrophobic solid material, was used as a control for the settlement test. Agarose and κ-carrageenan gels were used as natural polymer controls. The results demonstrated that fewer cyprids settled on any of the gel surfaces, compared with PS.

Materials and methods

Reagents

Acrylamide (AAm) (Junsei Chemicals, Tokyo, Japan) was purified by recrystallization from chloroform. 2-Hydroxyethylmethacrylate (HEMA) and 2-hydroxyethylacrylate (HEA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). *N*,*N*′-dimethylacrylamide (DMAAm), poly (vinyl alcohol) (PVA) (MW 95,000) and κ-carrageenan were purchased from Wako Pure Chemicals (Osaka, Japan). Agarose was purchased from Sigma-Aldrich (USA). Sodium *p*-styrene sulfonate (NaSS) (Tokyo Kasei Kogyo, Tokyo, Japan) was purified by recrystallization from ethanol and dried at 25°C *in vacuo*. 2-Acrylamide-2-methylpropanesulfonic acid (AMPS) was provided as a courtesy by Toagosei (Tokyo, Japan), and its sodium salt (NaAMPS) was obtained by neutralization of AMPS with sodium hydroxide in ethanol and purified by recrystallization from acetone. Acrylic acid (AAc)

(Kanto Chemicals, Tokyo, Japan) was distilled at a reduced pressure. *N*,*N*-dimethylaminopropylacrylamide, methylchloride quarternary (DMAPAA-Q) and *N*,*N*-dimethylaminoethylacrylate, methylchloride quarternary (DMAEA-Q) were purchased from Ko-jin Co. Ltd. (Tokyo, Japan). *N*,*N*′-methylenebis(acrylamide) (MBAA) (Tokyo Kasei Kogyo, Tokyo, Japan) as a crosslinking agent was purified by recrystallization from ethanol. 2-Oxoglutaric acid (Wako Pure Chemicals, Osaka, Japan), an initiator of free radical polymerization, was used as purchased. The precursor of PDMS (Silpot 184™) and the crosslinker of PDMS (catalyst of Silpot 184) (Dow Corning Toray Co., Ltd.) were used as purchased.

Gels for the settlement test

Single network gels by radical polymerization

PAAm, PDMAAm, PHEMA, PHEA, PNaAMPS, PAMPS, and PNaSS gels were synthesized by radical polymerization. One mole per litre of monomer in aqueous solution containing 4 mol% MBAA (as a crosslinker) and 0.1 mol% 2-oxoglutaric acid (as an initiator) in a glass reaction cell were purged with argon gas to eliminate the inhibition effect of oxygen on polymerization. Each solution was irradiated with UV light (wavelength 365 nm) for 10 h. PDMAPAA-Q and PDMAEA-Q gels were polymerized from a 2 mol l^{-1} aqueous monomer solution containing 8 mol% MBAA and 0.1 mol% 2-oxoglutaric acid, under the same conditions of purging and UV irradiation as described above (Chen et al. 2005).

PVA gel

Physically crosslinked PVA gel was prepared through a freezing and thawing method from a prescribed 10 wt% PVA aqueous solution. The solution was prepared by heating a mixture of PVA in an aqueous medium for 1 h at ~90°C. After the heating step, the PVA solution was cast between glass plates. PVA gel was obtained by seven cycles of freezing (−40°C) and thawing (25°C) (Kagata et al. 2002).

DN gels

PAMPS/PAAm DN and PAAc/PAAm DN gels were synthesized using a two-step sequential network formation technique (Gong et al. 2003). As the first step, PAMPS or PAAc gel (first network) was synthesized from a 1 mol l^{-1} monomer aqueous solution containing 4 mol% MBAA and 0.1 mol% 2-oxoglutaric acid by radical polymerization as described above. The gel was immersed in a 2 mol l^{-1} AAm aqueous solution containing 0.1 mol% 2-oxoglutaric acid for at least 1 day until equilibrium was reached. After the swollen gel was taken out of the AAm solution, the second network (PAAm) was subsequently polymerized in the presence of the first network gel (PAMPS or PAAc) under irradiation of UV light for 10 h between two plates of glass.

PDMS elastomer

PDMS elastomer was prepared according to commercially available product instructions. After degassing, a mixed solution of the precursor and crosslinker in weight ratios of 1.5, 2.5 and 3.5% was poured between glass plates separated by a silicone spacer. Polymerization was then carried out at 60°C for 4 h (Patra et al. 2008).

Natural polymer gels

Agarose and κ-carrageenan gels were prepared by the conventional method. The dissolving solution of autoclaved seawater and polysaccharide powder in a weight ratio of 10% was stirred for 15 min at 90°C. The hot polymer solution was directly poured into a PS well and cooled for 30 min at 6°C in a refrigerator (Gong et al. 2000).

After gelation

After gelation, gels were immersed in a large amount of autoclaved seawater for sufficient time to remove residual chemicals (usually 1 week) and the autoclaved seawater was changed once or twice each day. The resulting thickness of the gels was 0.5–2.0 mm. The PHEMA gel was opaque; the PAAm, PHEA, agarose, and PAAc/PAAm DN gels were semi-transparent and colorless; the PDMAPAA-Q gel was semi-transparent and yellowish and the others were transparent.

Evaluation of the elastic modulus of gels

The elastic modulus of the gels was evaluated using a compression test with a tensile–compressive tester (Tensilon RTC-1310A; Orientec, Co). For the compression test, samples were cut into a disc (10 mm diameter, 0.5–2.0 mm thickness) and compressed with two parallel metal platens connected to a load cell at a strain rate of 10% min^{-1} at room temperature. The elastic modulus was determined by the average slope of the stress–strain curve over the strain ratio range of 0–0.1.

Cyprid preparation

The *B. amphitrite* cyprid larvae were cultured according to standard procedures (Yoshimura et al. 2006).

Briefly, adult *B. amphitrite* brood stocks were maintained in plastic 10-l aquaria with aeration at a controlled temperature of 25°C, and were fed a daily diet of naupliar larvae of the brine shrimp *Artemia salina*. Adults maintained under these conditions spontaneously released nauplii within 30 min to a few hours upon changing the seawater. To collect photopositive nauplii, a concentrated light source was provided without aeration. Nauplii were collected by pipette and transferred to a beaker at a density of 3 larvae ml^{-1} and fed daily with the diatom *Chaetoceros gracilis*. The concentration of diatoms in the beaker was kept at 10×10^4 cells ml^{-1} at a temperature of 25°C with a photoperiod of 8 h light and 16 h dark. To prevent bacterial growth, a mixture of streptomycin and penicillin was added to the beaker at the beginning of the culture. When >60% of the larvae had reached the cyprid stage, usually after 4–5 days, the culture was filtered through plankton sieves to separate the cyprids from the nauplii. Cyprids were kept at 6°C in the dark in autoclaved seawater before settlement assays were carried out. Autoclaved seawater (120°C, 20 min) was used in all experiments.

Settlement assay

Assay conditions

Settlement assays were conducted according to standard procedures (Rittschof et al. 1992). Gel disks were placed on the bottom surface of a 6-well or 24-well PS multiplate (Nunclon). After sterilization by autoclaving (120°C, 20 min), gel disks were punched out of the gel plate by a hole-punch with a radius of 35 mm (used for the 6-well plate) or 15 mm (used for the 24-well plate); the thickness of the gel was several millimeters. The well wall surface of the entire well-plate was bare PS. Wells with a bare PS bottom surface were also used as a control. Then, 2 ml (6-well) or 0.5 ml (24-well) of autoclaved seawater containing 50 ± 10 cyprids stored for 2 days at 6°C in the refrigerator were poured into each well. The area ratio of the well bottom to the wall was about four (6-well) or one (24-well). Larvae-loaded multiplates were cultured in an incubator (LPH-100S; NK System, Japan) and held at a temperature of 25°C with a photoperiod of 8 h light under a cool white fluorescent lamp and 16 h dark for 5 days.

Procedure

Cyprid larvae from *B. amphitrite*, in the stage that explores suitable surfaces for settlement, were used for all the settlement tests. Cyprids that had metamorphosed into acorn barnacle were defined as 'settled' in this study, as schematically illustrated in Figure 2a.

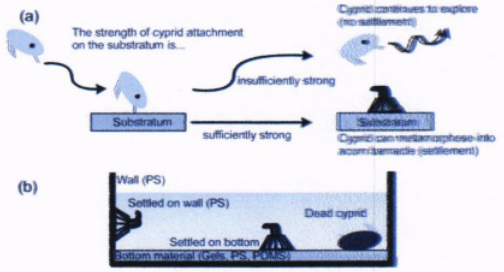

Figure 2. Cyprids metamorphosed into acorn barnacles were defined as 'settled' (a). The terms 'settled on bottom', 'settled on wall (PS)', and 'dead cyprid' used in this study are schematically illustrated in (b).

The number of cyprids settled on the bottoms of the wells consisting of various kinds of gels (as well as PS), on the wall surface of PS, and the number of dead cyprids (shown in Figure 2b) were counted using a stereomicroscope (SZX-12; Olympus, Japan) after exposure for 1, 3, and 5 days. The number of settled and of dead cyprids on each day was averaged over at least three wells.

Results and discussion

Figure 3a-1 shows the number and percentage settlement on the bottom surfaces covered with various kinds of gels and on bare PS. Because the settlement tests were performed in several different batches, the numbers and percentage settlement on the gels are shown as separate figures, with controls (PS) corresponding to each batch. The number of settlements for all tests was averaged over at least three wells. After 5 days, 53–75% of the cyprids settled directly on the PS bottom surface. In contrast, all types of gels showed much less settlement than that on PS. Especially, no settlement at all was observed on the PHEMA, PnaSS and PVA surfaces, or on the agarose gels, even after 5 days. On the other hand, the κ-carrageenan gel, which is made from natural polymer, had the highest settlement number (about half that of the PS control). Figure 3a-2 shows that most of the cyprids settled on the walls of the PS wells instead of on the bottom gel surfaces. The settlement direction was not found to be important for settlement selectivity, as the settlement ratio per unit area of bottom (PS) to wall (PS) was one on average 0.4–1.8. Furthermore, only a few of the cyprids were dead (number dead/number of total cyprids fed × 100% = 0.9–10.4%), which was similar to that of the controls (0.8–6.8%) (Figure 3a-3), indicating that none of the gels was toxic to the cyprids.

Because cyprids arrive at surfaces by random swimming, it is difficult to consider that they recognize

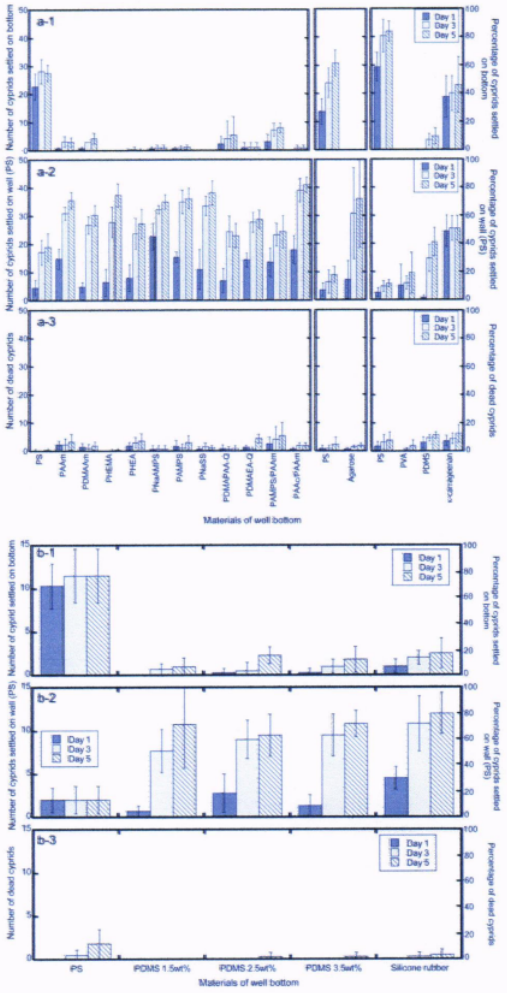

Figure 3. The number of (a-1) cyprids settled on the bottom of wells covered with PS, PAAm, PDMAAm, PHEMA, PHEA, PNaAMPS, PAMPS, PNaSS, PDMAPAA-Q, PDMAEA-Q, PAMPS/PAAm DN, PAAc/PAAm DN, PVA gels, and PDMS elastomer; (a-2) cyprids settled on the PS wall of wells; (a-3) dead cyprids in wells, after 1, 3, and 5 days exposure. The area ratio of bottom to wall was about four (6-well multiplate). Each set of data in three different frames was obtained from three different experimental runs. The number of (b-1) cyprids settled on the bottom of wells with PS and PDMS with different elastic moduli, (b-2) cyprids settled on the PS walls of wells, and (b-3) dead cyprids in wells, after 1, 3, and 5 days exposure. The area ratio of bottom to wall was about one (24-well multiplate). Error ranges are SDs over at least three wells in all the tests.

a particular surface at the initial landing. Instead, they will land on all surfaces with the same probability. Therefore, lower or even no settlement on hydrogels is most likely due to the 'ease of release' of initially attached cyprids, which is the main determinant of the conversion from random cyprid contact and surface exploration to settling and metamorphosis. It should be noticed that the 'settlement' defined in this work required sufficiently strong and persistent attachment of the surface-contacting cyprid antennules tips (with cement) to trigger metamorphosis of the organism to the acorn barnacle form (Figure 2a). When the strength of adhesion is less than the contractile force of the retracting cyprid antennules, cyprids do not 'settle' but wander aimlessly and eventually die without undergoing metamorphosis.

As shown in Figure 3a, although all the gels showed an AF effect, settling was different for different gels. To clarify the main factor that influences the AF performance of gels, the results are first discussed in terms of their electric nature. In the case of the neutral gels, cyprids could not settle at all on the PHEMA and PVA gels, whereas there was slight settlement on the PDMAAm (6.4%), PAAm (5.8%), and PHEA (0.4%) gels. In the case of the anionic gels, the PNaSS gel had no settlement, although slight settlement was observed on the PAMPS (2.0%) and PNaAMPS (1.3%) gels. Low cyprid settlement on the cationic gels, such as the PDMAPAA-Q (13.5%) and the PDMAEA-Q (1.7%) gels, was also observed. There was no clear relationship between the electric nature of the gels and settlement. These findings therefore suggest that the electric nature of the gels had no significant effect on their AF performance against barnacles. Furthermore, in seawater, which has an ionic strength of 0.7 mol kg^{-1}, the charge effect would be screened out and all the gels would behave like neutral surfaces.

Next, the effect of surface energy on cyprid settlement is discussed. It would be expected that the hydrophilic surface of hydrogels might affect the settlement of cyprids. It has been reported that the surface free energy of hydrogels is slightly lower than, but quite close to, that of water (about 72.1 mN m^{-1}) (Andrade et al. 1979). For example, the surface free energy of the PAMPS gel is reported as 67.1 mN m^{-1} (Szabo et al. 2000). However, similar AF behavior was also observed toward soft hydrophobic surfaces such as the PDMS elastomer (19.8–20.4 mN m^{-1}) (Ishii 2001), which is lower in surface free energy than PS (40.0–44.8 mN m^{-1}) (Ishii 2001). The relationship between the surface energy of several substrata and the retention strength of adhered biofouling organisms has been reported (Baier 2006). The report shows that the retention strength of the attached biofouling organisms is minimal in the critical surface tension range between 20 and 30 mN m^{-1}, and after reaching a peak at 60 mN m^{-1}, it substantially decreases again with the increase in the critical surface tension. The present

observation of low retention strength on silicone elastomer and on hydrogels is in agreement with this report, that is, 'easy-release' hydrogel and 'easy-release' PDMS silicone elastomer, which are opposite in the hydrophilic/hydrophobic qualities, share the 'θ surface' quality.

As mentioned above, on the DN gel composed of negatively charged PAMPS and neutral polymer PAAm networks (PAMPS/PAAm DN gel), cyprid settlement was greatest (16.1%) among all the gels. On the other hand, on the DN gel composed of weakly negatively charged PAAc and a neutral polymer PAAm network (PAAc/PAAm DN gel), low cyprid settlement (1.5%) was observed. These results suggest that interpenetrating polymer network structures, such as DN gels, are not important for cyprid settlement behavior.

Next, the effects of the water content and the elasticity (E) of the gels, two key parameters for soft and wet gels, are discussed. The water content of a gel is usually expressed in terms of swelling degree q, which is defined as the volume ratio of the swollen gel to the dried gel. According to the scaling theory, the elastic modulus E and the swelling degree q obey a scaling relationship of E–q^{-3} for a non-charged gel with its partial chains in a Gaussian distribution (de Gennes 1979).

The relationships between the elastic moduli and the swelling degrees of hydrogels with various chemical structures are shown in Figure 4. Figure 4 shows that the E of gels substantially decreases with an increase in the q, approximately obeying the scaling relationship of E–q^{-3}, even for gels with negative or positive charges. This also indicates that the high ionic strength of seawater would screen out all of the charge effects and all of the gels would behave approximately like neutral surfaces.

The number of cyprids settled on the bottom after a 5-day exposure was replotted against the elastic modulus (Figure 5a) and the swelling degree (Figure 5b) of the gels. Figure 5a shows that for PHEMA, PHEA, PNaAMPS, PAMPS, PNaSS, PDMAEA-Q, PAAc/PAAm DN, agarose, and PVA gels, a relatively low settlement number was observed over a wide range of elastic moduli. On the other hand, the relative settlement number for PAAm, PDMAAm, PDMAPAA-Q and PAMPS/PAAm DN gels increased with an increase in the elastic modulus of the gel. These results indicate that the tested hydrogels can be categorized into two groups, one (Group 1) showing very low cyprid settlement, regardless of elastic modulus, and the other (Group 2) showing relatively high cyprid settlement, increasing with the elastic modulus of the gel.

Figure 5b shows that on gels of Group 2, settlement numbers decreased with an increase in the swelling degree of the gel; however, the gels of Group 1 did not depend on q. At the present time, it is not apparent why the κ-carrageenan gel exhibited the most settlement.

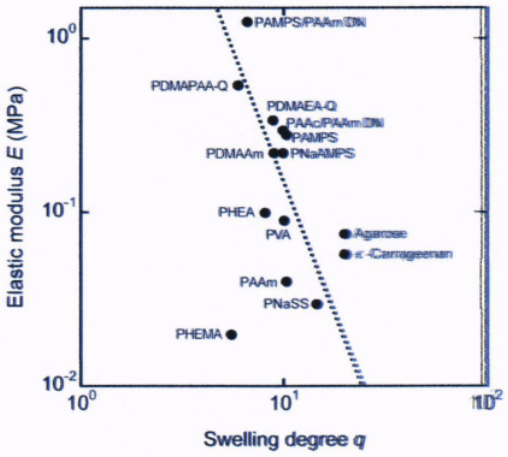

Figure 4. The relationship between the elastic modulus (E) and the swelling degree (q) of the hydrogels. The broken line indicates the scaling relationship of E–q^{-3}.

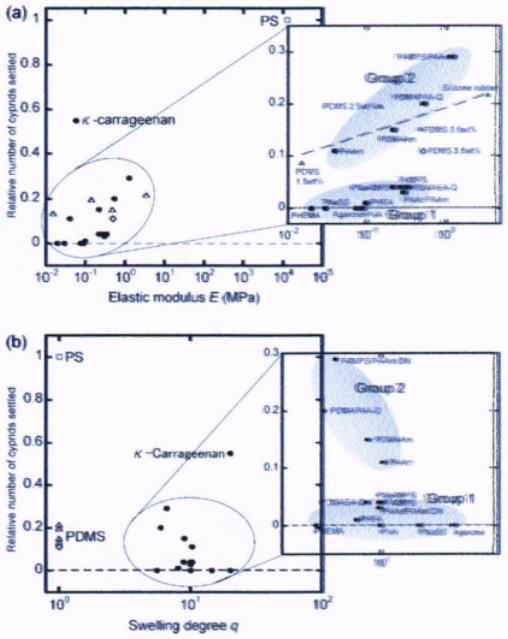

Figure 5. The relationship between the numbers of cyprids settled on the bottoms of wells after 5 days exposure and the (a) elastic modulus, and (b) swelling degree of bottom materials. ●=hydrogels; ◇ = PDMS (first run); Δ = silicone elastomer (PDMS) with different elastic moduli; □ = PS.

Because E varies with q, it is not clear whether the E or the q plays the primary role in determining cyprid settlement on hydrogels of Group 2 (PAAm, PDMAAm, PDMAPAA-Q, and PAMPS/PAAm DN gels), as shown in Figure 5. To distinguish between the effects of E and q, the results for the hydrogels were compared with that of a non-water swollen elastomer, PDMS, at various elastic moduli, as well as with hard and dry PS. The elasticity of PDMS can be controlled by changing the weight ratio of the crosslinker during synthesis. For solid materials that do not hold water, such as PS and PDMS, the swelling degree equals one for any elastic modulus, permitting independent investigation of the elasticity effect.

Five hundred microlitres of autoclaved seawater containing ~15 cyprids were loaded into wells with their surfaces covered by PDMS of various elastic moduli. The surface ratio of the bottom (PDMS) to the wall (PS) was about one. A well without PDMS was used as a control. The results shown in Figures 3b and 5 indicate that cyprid settlement on the bottom surface tended to decline with a decrease in the elasticity of the PDMS. These results indicate that elasticity does influence cyprid settlement and suggest that the increase in settlement observed on gels of Group 2 might be due to the change in elasticity rather than the swelling degree of the gel.

In conclusion, the results of this study demonstrate that fewer cyprids were retained on all hydrogels compared with PS. This low adhesion of cyprids is independent of surface hydrophilicity (hydrogels) or hydrophobicity (PDMS elastomer) and of surface charge. From their AF behavior, hydrogels can be categorized into two groups. Group 1 (PHEMA, PHEA, PNaAMPS, PAMPS, PNaSS, PDMAEA-Q, PAAc/PAAm DN, agarose, and PVA gels) showed very low settlement over a wide range of elastic modulus. On the other hand, Group 2 (PAAm, PDMAAm, PDMAPAA-Q, and PAMPS/PAAm DN gels) showed increased settlement of cyprids with an increase in the elastic modulus (or decrease in the swelling degree) of the gel. In the latter case, the elasticity, rather than the swelling degree, may be the important factor for cyprid settlement, as suggested by the elasticity effect observed for PDMS. As a result, the authors believe that these gels could be good candidates for an environmentally benign AF system. In particular, PAMPS/PAAm DN and PVA gels are strong enough to undergo a long-term test in the marine environment. The authors intend to report, in a separate paper, the results of an AF test with several hydrogels, including PAMPS/PAAm DN and PVA gels, in the marine environment.

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