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ACTIVATION OF LYMPHOCYTE PROLIFERATION BY BORONATE-CONTAINING POLYMER IMMOBILISED ON SUBSTRATE: THE EFFECT OF BORON CONTENT ON LYMPHOCYTE PROLIFERATION

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Abstract

This study demonstrates that boronic acid-containing polymers coated onto solid support function as synthetic mitogens for mouse lymphocytes. The polymer was synthesized by radical copolymerization of 3-acrylamidophenylboronic acid with dimethylacrylamide (poly(AAPBA-DMAA)). The boronic acid in the trigonal form in the copolymer activated lymphocytes, probably by crosslinkage to glycoprotein moieties on the plasma membrane surface, as in the case of lectin stimulation. A higher concentration of phenylboronic acid on the copolymer surface resulted in greater activation of lymphocytes, suggesting that the number of phenylboronic acid residues per unit area may be a crucial factor in lymphocyte proliferation. The proliferative response of lymphocytes was also affected by the surface wettability, probably due to a difference in the flexibility of polymer strands at the cell-polymer interface.

Key Words: Lymphocyte, phenylboronate, boronic acid-containing polymer, artificial lectin, mitogen.

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Introduction

Lymphocytes consist of various subpopulations with distinctive functions, which play important roles in immune responses (Marchalonis, 1988). Activation and proliferation of these subpopulations can be achieved by treating them with mitogens. The best-known group of mitogens are sugar-binding proteins called lectin. Lectins have been widely used for cell separation and functional analysis owing to their specific binding properties to a particular sugar sequence expressed on the surface of the plasma membrane (Sharon and Lis, 1972, 1998). Lectin treatment occasionally induces lymphocytes with high anti-tumor activity, and thus it is expected to be useful for cancer therapy (Mulé *et al*., 1984; Mulé and Rosenberg, 1985). However, lectins of plant origin have antigenicity in humans (Franz *et al*., 1983; Kulkarni et al., 1998; Leist and Wendel, 1998), and their stability are not always adequate for use in immunotherapy and medical engineering, including tissue engineering and drug delivery (Kijne, 1996). These problems may be resolved through the development of a completely synthetic polymer with lectin-like function (Miyazaki *et al*., 1993).

We previously synthesized a novel water-soluble polymer with lectin-like function (artificial lectin) by introducing phenylboronates, as sugar-recognizing moieties, into the side-chain of poly-N,N-dimethylacrylamide (Miyazaki, 1993). Phenylboronates were chosen because they can easily form reversible covalent bonds with polyol compounds, including sugars (Conner and Bulgrin, 1967; Barker *et al*., 1973). At physiological pH, phenylboronates form an appreciably stable complex with sialic acid (Neu5Ac), a characteristic anionic carbohydrate on the surface of the plasma membranes (Otsuka *et al*., 2003). A prepared polymer induced appreciable proliferation of murine splenic lymphocytes with increased expression of IL-2 receptor on their surface, presumably due to the selective binding to Neu5A components on the lymphocyte membrane (Uchimura *et al*., 2001). Increased proliferation of lymphocytes showing cytotoxicity against YAC-1 cells

was achieved by concurrent addition of IL-2 with the boronate-containing polymer in the medium, suggesting that this polymer may be an effective immuno-adjuvant for the induction of lymphokine-activated killer (LAK) cells (Uchimura *et al*., 2001).

Moreover, compared with its soluble form, immobilized natural lectin on solid matrix induced even greater proliferation of cells, including lymphocytes (Greaves and Bauminger, 1972). For example, a polymer substrate with immobilized Ulex I lectin was reported to have a high ability to induce adhesion and successive proliferation of vascular endothelial cells (Parhizgar *et al*., 1987), suggesting that lectin-immobilized substrate may be useful in the field of tissue engineering. Enhanced uptake of particles by gastrointestinal epithelial cells has been achieved by immobilizing a certain group of lectins (Tomato, Mycoplasma) on the particle surface (Irache *et al*., 1994), which may become a useful approach for oral delivery of vaccine and other physiologically active compounds. Inspired by these results, we performed a systematic study of the interaction of cells, including lymphocytes, with substrates coated with phenylboronate-containing polymer, to obtain an important basis for the development of boronate-based solid-support which might be useful for the modulation of cellular functions (Kataoka *et al*., 1989; Jozefowicz and Jozefowicz, 1990). When bovine aortic endothelial cells were cultured on substrate coated with phenylboronate-containing polymer they adhered together and proliferated, spontaneously forming a capillary structure (Aoki *et al*., 1995).

Here, we report the use of a boronic acid-containing solid substrate for the activation and proliferation of lymphocytes through the binding to carbohydrates expressed on their plasma membrane. The substrate was composed of N,N-dimethylacrylamide (DMAA) and 3-acrylamidophenylboronic acid (AAPBA). An important aim of the study was clarification of the effects of the surface properties of the substrate, including the surface boron concentration and wettability, on the proliferative response of lymphocytes.

Experimental

Materials

DMAA (Wako Pure Chemical Co. Ltd., Tokyo, Japan) was purified by distillation under reduced pressure. AAPBA (Wako) and 2, 2'-azobis(2, 4-dimethylvaleronitrile) (V-65; Wako) were recrystallized from ethanol and dried thoroughly *in vacuo* at room temperature. N-Phenylacrylamide (PA; Polyscience Inc., Warrington, PA, USA) was obtained commercially and used without further purification. Other reagents and solvents were purified by standard methods.

Preparation of Polymer Samples

Copolymers of DMAA with AAPBA (DB copolymer) with varying composition were prepared by radical copolymerization as follows: various ratios of DMAA and AAPBA were dissolved in ethanol, and V-65 was added to initiate copolymerization. The reaction mixture was stirred in a sealed glass ampoule *in vacuo* at 40°C for 30 min. Polymerization was quenched by pouring the reaction mixture into excess diethyl ether. The precipitated DB was filtered and dried thoroughly *in vacuo*. As a control polymer sample without phenylboronic acid moieties, copolymer DP was prepared by radical copolymerization of DMAA with PA, following a procedure similar to that used to prepare DB but at a reaction temperature of 45°C and with different concentrations of the monomers and initiator (Ikeya *et al*., 1998).

Compositions of DB and DP were determined by ^{1}H-NMR (EX-400, JEOL Ltd., Tokyo, Japan) in DMSO-d_6 at 80°C. DB and DP were designated as DB-X and DP-X, where X is the mol% of AAPBA and PA, respectively. Their weight-averaged molecular weights were determined by static light scattering (DLS-700, Otsuka Electronics Co. Ltd., Osaka, Japan) in ethanol.

Determination of Surface Boron Contents of DB-X Film by X-ray Photoelectron Spectroscopy (XPS)

The elemental composition of the surface of a DB-X film on polystyrene disks (diameter: 8 mm) were determined using XPS to quantify the phenylboronic acid moieties in the outermost layer of the films. The samples were prepared by casting DB-X polymers in ethanol onto the polystyrene disks. Spectra were acquired using an AXIS-MS electron spectrometer (Kratos Analytical Ltd., Manchester, UK), and the take-off angle was 90°. Five survey scans and twenty detailed scans of C1s and B1s were recorded for each sample. The area ratio of B1s peak (binding energy: 200 eV) to C1s peak (binding energy: 295 eV) (B/C ratio) was used as an index of the boron content on the copolymer surface.

Contact angle measurement (Otsuka *et al*., 2000a,b)

The variation of static wettability of the surface covered with 0.5% DB and DP copolymers by buffered solutions

Table 1. Characterization of polymer samples.

code	feed composition (molar ratio)			yield (%)	molar composition of obtained polymers[a)]			M_w[b)] (X10^5)
	DMAA	AAPBA	PA		DMAA	AAPBA	PA	
DB-17.5	0.875	0.125	-	30.3	0.825	0.175	-	0.9
DB-33	0.7	0.3	-	70.4	0.67	0.33	-	3.0
DB-55	0.5	0.5	-	81.5	0.45	0.55	-	4.4
DP-37	0.7	-	0.3	19.7	0.63	-	0.37	0.6

a) Copolymer composition was determined by ^{1}H-NMR.
b) Mw was determined by static light scatting in EtOH.

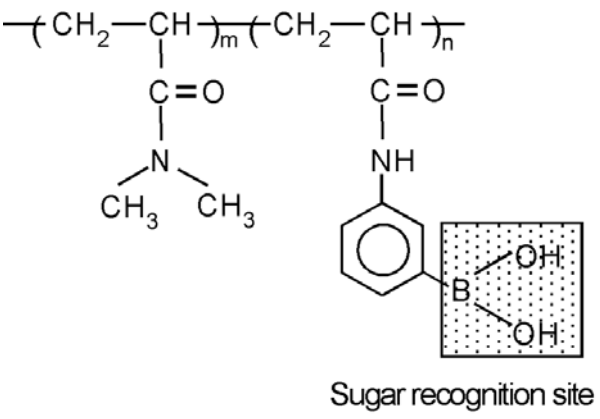
in the pH range of 5-11 was evaluated using a contact angle meter (CA-W: Kyowa Kaimen Kagaku Co. Tokyo, Japan). The buffers were CH_3COOH/NaOH/NaCl (pH 5), phosphate buffer solution (6.0<pH<8.0), sodium *p*-phenosulfonate/NaOH buffer solution (8.0<pH<9.0), and $NaHCO_3$/NaOH/NaCl buffer solution (>pH9.0), all with an ionic strength of 0.15, maintained by adding an appropriate amount of NaCl. The captive bubble technique was used, whereby the sample film was immersed in water maintained at 25°C and a small air bubble was placed under the film using a curved needle (Fig. 3). The contact angle of each film sample was measured at 15 or more spots, and the values were averaged.

Lymphocyte Preparation

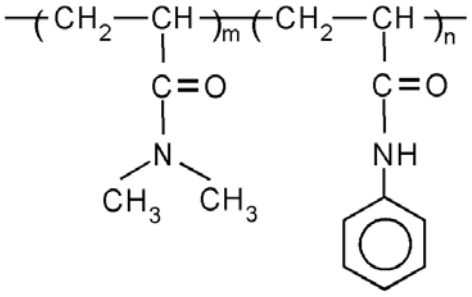
Mouse lymphocytes were obtained under sterilized conditions from spleens of 5-week-old male AKR mice (Sankyo Laboratories Co. Ltd., Tokyo, Japan) sacrificed by dislocation of the cervical vertebrae (Miyazaki *et al*., 1993). The spleens were immersed immediately into 25 mM HEPES (N-2-hydroxyethyl-N'-2-sulfoethyl-piperazine)-buffered RPMI 1640 medium in Petri dishes. The lymphocytes were released by flushing the spleens several times with RPMI 1640 medium using a 10-ml disposable plastic syringe fitted with a 26-gauge needle. The lymphocyte suspension was filtered through a 70-µm mesh nylon net to remove debris, and centrifuged at 200*g* for 5 min at room temperature. The lymphocyte pellets were then mixed gently with Tris-buffered 0.83% (w/w) NH_4Cl to lyse contaminating erythrocytes (Boyum, 1968), and washed twice with RPMI 1640 medium. The number of lymphocytes in the suspension was counted using a Coulter counter (model ZM, Beckman Coulter Inc., Fullerton, CA, USA), and adjusted to the desired cell concentration by addition of 25 mM HEPES-buffered RPMI 1640 medium.

Lymphocyte Proliferation Assay

The copolymer dissolved to a certain concentration in ethanol was used to prepare the polymer coating on the inner surface of a flat-bottomed multi-well plate (Falcon 3042, Becton-Dickinson Co. Ltd., Franklin Lakes, NJ, USA). Each well was treated with the polymer solution and dried under argon at room temperature for 2 days and then *in vacuo* at 45°C for 1 day. Lymphocytes were suspended in 25 mM HEPES-buffered RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) at $2x10^6$ cells/ml, and 200 µl of this suspension was plated into a polymer coated multi-well plate. The lymphocytes were cultured at 37°C for 45 h in a humidified 5% CO_2 atmosphere. Then, 20 µl of ^{3}H-thymidine (Amersham-Pharmacia Biotech, Uppsala, Sweden) was added to the suspension and the lymphocytes cultured for a further 3 h. The lymphocytes were harvested onto a glass filter (Skatron Instrument Inc., Sterling, VA, USA), and their radioactivity was measured by liquid scintillation (Aloka Co. Ltd., Tokyo, Japan) using a scintillation cocktail (27 mM of 2, 5-diphenyloxazole and 1.1 mM of 4-bis(2-phenyloxazoyl)benzene in toluene).



poly(AAPBA-DMAA) (DB-X)



poly(PA-DMAA) (DP-X)

Figure 1. Structural formulas of copolymers of N,N-dimethylacrylamide (DMAA) with 3-acrylamidophenylboronic acid (AAPBA), shown as poly(AAPBA-DMAA) (DB copolymer), and DMAA with N-Phenylacrylamide (PA), shown as poly(PA-DMAA) (DP copolymer). The value of x denotes the molar composition of AAPBA for poly(AAPBA-DMAA) and PA for poly(PA-DMAA), respectively.

Results and Discussion

Characterization of Substrata Coated with Boronate-containing Copolymer

Fig. 1 shows the structural formulae of DB and DP. Table 1 lists their weight-averaged molecular weights, chemical structure, and compositions. The contents of AAPBA and DP units in the copolymer were controlled so as to be water-insoluble in the experimental conditions.

The elemental binding states and compositions of polystyrene surfaces coated with DB copolymer were examined by survey scans and high resolution C1s and B1s of XPS analysis, and the results are summarised in Fig. 2 as a function of the coating concentration of the copolymer with the ratio of core-level photoelectron emissions for B1s to C1s. This ratio is used as an index of the boron content on the copolymer surface. Obviously, the ratio increases with coating concentration and then reaches plateau value in the range of 4 to 6 irrespective of

the copolymer composition. The calculated B1/C1 ratios based on sensitivity factor (B:C=0.49:1) were 1.51 for DB-17.7, 2.49 for DB-33, and 3.68 for DB-55, respectively, suggesting that phenylboronic acid moieties tend to be enriched at the surface for all coatings, probably owing to their hydrophobic character. The surface boron content of DB increases with PBA composition.

pH-dependent change in contact angle of the charged surface determined by contact angle titration provides data on the dissociation state and concentration of charged functional groups on the surface, and correlates with material properties, at the macroscopic level, such as wettability and adhesion strength (Holmes-Farley *et al*., 1985; Holmes-Farley and Whitesides, 1986). Contact angle titration results have also been correlated with the degree of ionization determined directly by ATR-IR spectroscopy (Holmes-Farley *et al*., 1985), fluorescence spectroscopy (Holmes-Farley and Whitesides, 1986), and conventional titration (Holmes-Farley *et al*., 1985). Holmes-Farley *et al*. (1985) reported that the contact angle of water on polyethylene film derivatized with ionizable carboxylate groups changes systematically with the pH of the water, correlating nicely with the ionization degree of the carboxylate groups. However, this study was performed using dry films, and thus, the measured interface may not be in thermodynamic equilibrium with the water phase, resulting in hysteresis. To avoid this effect, the wettability of the boronated surface was estimated in buffer (I=0.15), providing information regarding the boronic acid content and its dissociation equilibrium on the surface at a given pH. Table 2 summarizes the contact angle titrations on the film samples coated with DB and DP copolymers, respectively, as a function of pH. As DB-17.5 was slightly water-soluble under alkaline conditions, contact angle titration was performed for DB-33 and DB-55. With the captive bubble technique shown in Fig. 3, the higher contact angle (θ) corresponds to the increased hydrophilicity. Table 2 clearly indicates that surfaces containing ionizable boronate groups (DB-33, DB-55) undergo an appreciable change in contact angle with pH: a higher contact angle is associated with higher pH. This is due to increased ionization of the boronate with higher pH, which induces increased water adsorption onto the polar and charged tetrahedral boronate group. The work

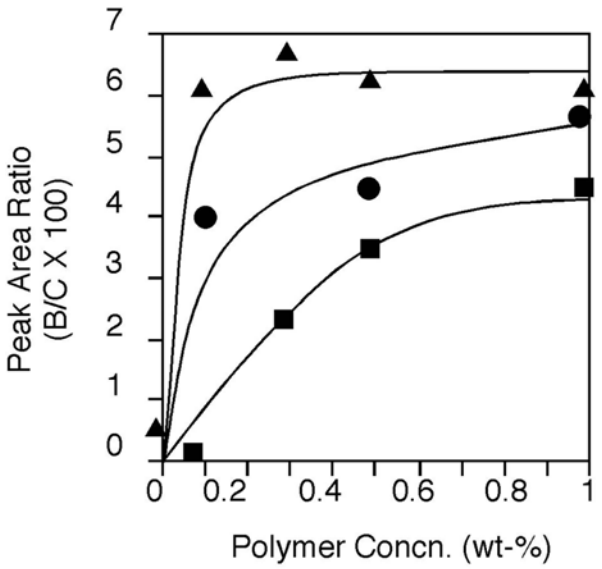


Figure 2. Phenylboronic acid content on the substrate coated with poly(AAPBA-DMAA) (DB copolymer) examined by survey scans and high resolution C1s and B1s of XPS analysis, which is summarised as a function of the coating concentration of the copolymer with the ratio of core-level photoelectron emissions for B1s to C1s. (■) DB-17.5, (●) DB-33, and (▲) DB-55.

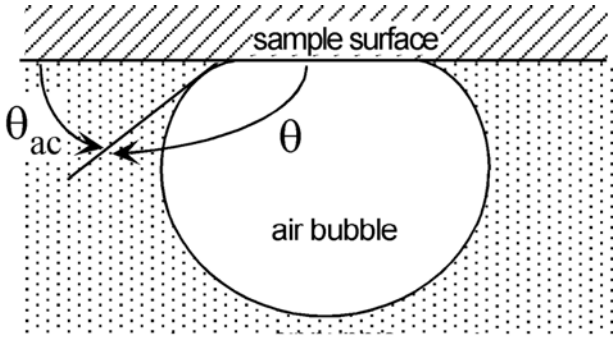


Figure 3. Schemaic representation of contact angle measurement in water. θ (degree) is the angle between the solid surface and the air bubble interface measured inside the bubble. θ is related to the actual contact angle θ_{ac} by $\theta=180-\theta_{ac}$. The higher contact angle (θ) corresponds to the increased hydrophilicity (smaller value of θ_{ac}).

Table 2. Contact angle titration for interfaces with and without phenylboronic acid groups.

	DP-37		DB-33		DB-55	
pH	θ	cos(180-θ)	θ	cos(180-θ)	θ	cos(180-θ)
4	117.7	0.46	119.5	0.49	110.5	0.35
5	118.5	0.48	121.1	0.52	111.3	0.36
6.2	118.8	0.48	124.4	0.56	113.0	0.39
7.4	118.9	0.48	135.7	0.72	123.6	0.55
8.6	117.1	0.46	143.2	0.80	148.4	0.85
9.8	118.8	0.48	150.0	0.87	157.9	0.93
11	118.8	0.48	151.0	0.87	158.5	0.93

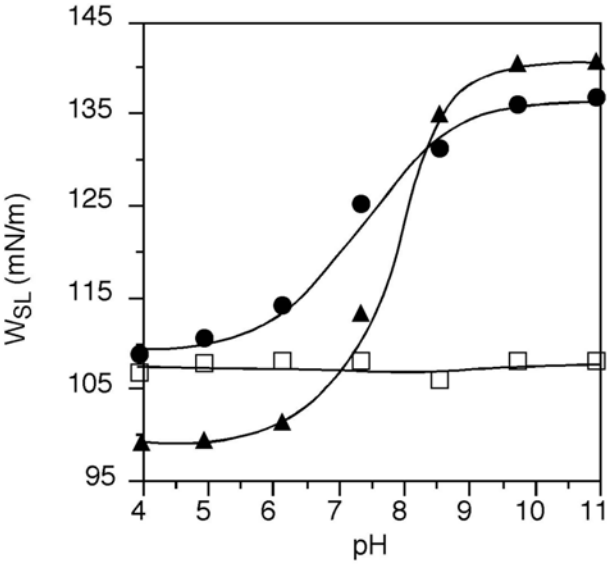


Figure 4. Contact angle titration for interfaces with and without phenylboronic acid groups, showing the work of adhesion between the contacting solid--liquid interface, W_{SL}, calculated as a function of pH. (●) DB-33, (▲) DB-55, and (□) DP-37.

of adhesion between the contacting solid-liquid interface, W_{SL}, was then calculated as a function of pH to get more insight into the system (Fig. 4). As shown in eqs. (1) and (2), W_{SL} includes a substantial contribution from both polar and hydrogen bonding components.

$$W_{SL} = \gamma_L(1+\cos\theta) \quad \text{(Young - Dupre equation)} \quad (1)$$
$$= \gamma_L + \gamma_S - \gamma_{SL}$$
$$= 2\sqrt{\gamma_L^d \gamma_S^d} + 2\sqrt{\gamma_L^p \gamma_S^p} + 2\sqrt{\gamma_L^h \gamma_S^h} \quad \text{(geometric mean approximation)} \quad (2)$$
$$= W_{SL}^d + W_{SL}^p + W_{SL}^h$$

where γ^d, γ^p, and γ^h are components of the surface free energy γ, arising from the dispersion force, the polar force, and the hydrogen-bonding force, respectively. Dipole and hydrogen bonding characteristics should be important because the hydration of phenylboronic acid through ionization on the DB surface is the main factor in wettability, which influences lymphocyte interaction. Fig. 4 shows that the W_{SL} of surfaces coated with DB copolymers is considerably dependent on pH , which is in agreement with the titration curve for 3-propionamidophenylboronic acid; PAPBA, a model compound of AAPBA units in DB copolymer (Hisamitsu, 1997). The change in W_{SL} with pH was more significant for DB-55 than for DB-33, reflecting the higher concentration of boronate on the former surface (Fig. 2). This was also suggested by the higher W_{SL} value for DB-55 at pH 8.6, which is the p*K*a of the corresponding monomer (PAPBA) (Kataoka *et al.*, 1994). On the other hand, the surface coated with DP copolymer as control showed no change in W_{SL} as a function of pH. Accordingly, the equilibrium between the neutral phenylboronic acid and the anionic phenylboronate is indeed a key factor in the characteristics of the DB-coated surface.

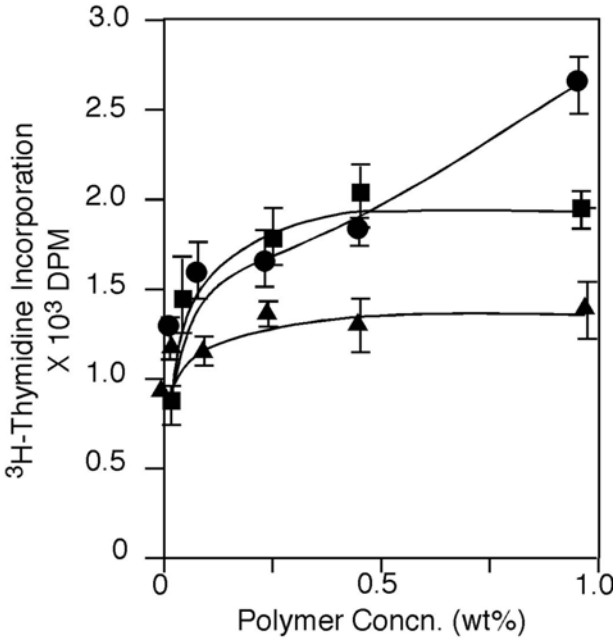


Figure 5. ^{3}H thymidine incorporation of lymphocytes stimulated by poly(AAPBA-DMAA) (DB copolymer) on the substrate. Mouse spleen cells ($4.0x10^5$ cells/well) were activated with varying concentration of poly(AAPBA-DMAA): (■) DB-17.5, (●) DB-33, and (▲) DB-55 on the substrate. Vertical bars represent S.E.M. of triplicate experiments.

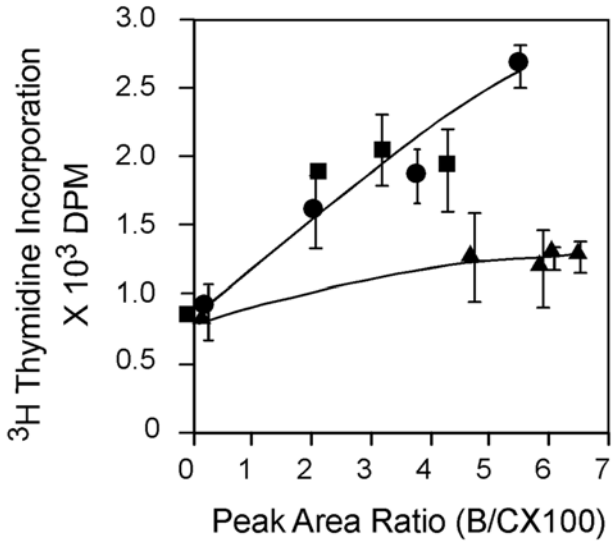


Figure 6. Effect of immobilized phenylboronic acid content on ^{3}H-thymidine incorporation of lymphocytes: (■) DB-17.5, (●) DB-33, and (▲) DB-55 on the substrate. Immobilized phenylboronic acid content was estimated from B/N area ratio in XPS spectra (take-off angle: 90°).

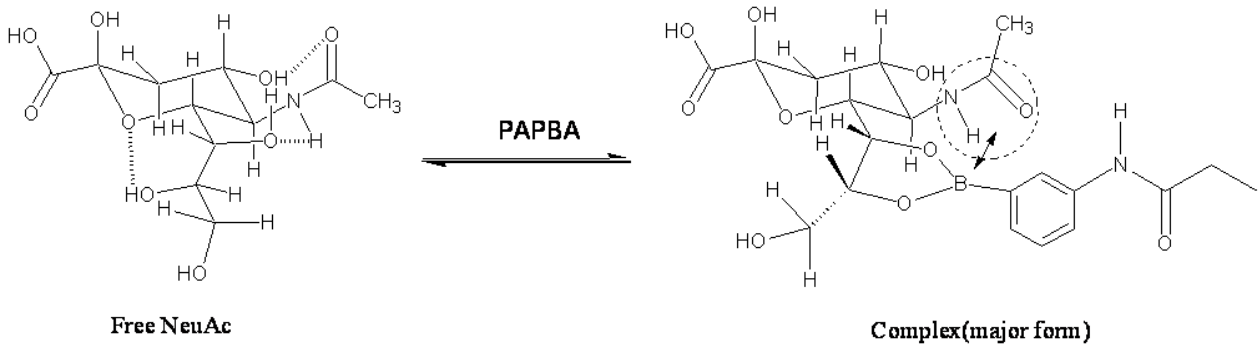


Figure 7. Conformational model for the Neu5Ac and the Neu5Ac/PAPBA complex, confirmed from NMR analysis at physiological pH7.4.

Lymphocyte Proliferation Assay

The feasibility of DB copolymer-coated substrates stimulating lymphocytes to proliferate was examined by monitoring the ^{3}H-thymidine uptake of lymphocytes after 48 h of cultivation in DB copolymer-coated wells. Figure 5 shows ^{3}H-thymidine incorporation into lymphocytes cultured in polystyrene wells coated with DB copolymers with varying boronate content. ^{3}H-Thymidine uptake increased with increasing concentration of the DB coating the wells, ^{3}H-thymidine uptake levelled off in the range of 0.5-1 wt-% on surfaces covered with DB-17.5 (■) and 55 (▲).

DP copolymer without phenylboronic acid moiety does not induce ^{3}H-thymidine uptake by lymphocytes (Uchimura *et al*., 2001). The essential role of phenylboronate moieties in lymphocyte proliferation was confirmed by our previous study demonstrating appreciable inhibition of boronate-induced lymphocyte proliferation by sorbitol, which is known to have a considerably high binding affinity to phenylborate compounds (Uchimura *et al*., 2001). Moreover Fig. 5 indicates that the extent of lymphocyte proliferation is dependent on the boron content in the copolymer. To get some insight into this observation, Fig. 5 was replotted as Fig. 6 in which the surface boron content had been determined by XPS.

With increasing amounts of boron on the surface, an appreciable increase in ^{3}H-thymidine uptake was observed for DB-17.5 and DB-33 surfaces, while no significant increase in the proliferation of lymphocytes was observed on the DB-55 surface, as shown in Figure 6. Consequently, at the same surface boron concentration, marked difference in the ability to proliferate lymphocytes was observed between the surfaces with low (DB-17.5 and DB-33) and high (DB-55) boron contents. Our previous study confirmed the anomalously high equilibrium constant (K) for Neu5Ac binding to PAPBA at physiological pH7.4 through the formation of the characteristic trigonal borate complexes, as schematically shown in Fig. 7 (Otsuka *et al*., 2003). Presumably, the appreciably higher proliferation ability of DB-17.5 and DB-33 compared with DB-55 may be correlated to their higher hydrophilic surface at pH7.4. A less hydrated polymer chain of DB-55 is assumed to have lower ability to access the carbohydrate (Neu5A) moieties on the lymphocytes due to the limited conformational motion. On the other hand, the hydrophilic DB-17.5 and DB-33 coated surfaces contain higher amounts of associated water to induce chain flexibility, which appreciably contribute to lymphocyte recognition by the borate binding to the carbohydrate moieties on the lymphocyte plasma membrane.

Conclusion

Solid matrix coated with polydimethylacrylamide partially derived from phenylboronic acid (DB copolymer) was evaluated as a synthetic mitogen of lymphocytes. Phenylboronic acid moiety in the copolymer works as a recognition site of carbohydrates, presumably Neu5Ac, on the surface of lymphocytes through the formation of a stable ester linkage. The amount of substantial phenylboronic acid per unit area seems to be a determining factor in lymphocyte proliferation, and increased amounts of phenylboronic acid results in activation of more lymphocytes. The proliferative response of lymphocytes was further emphasized by increased surface hydrophilicity, probably because increased flexibility of the polymer strands at the interface provide more freedom in spatial matching of polymer-bound boronic acid to carbohydrate moieties expressed on the lymphocyte surface. The improved mitogenic activity by immobilization of the polymer on solid support suggests that lymphocytes may be activated by a column with immobilized phenylboronic acid-derivatized copolymer. This would probably be useful in the field of immuno-modulation and cellular engineering, where engineered biomaterials with cell-regulating functions are in strong demand.

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Discussion with Reviewers

Reviewer I: Apparently the authors have not realised that the hydrophilic range of contact angles involves angles from 0°-90° and the hydrophobic range angles from 90° to 180°. In Table 2 only contact angles >110° are listed i.e. the surface of the authors is very hydrophobic and shows a paradox behaviour in that at high pH, when boronic acid dissociates and a more hydrophilic surface is expected due to the generated charges (and to increased binding of water molecules, as the authors correctly state), the authors observe an increase in contact angles i.e. an increase in hydrophobicity! This paradox effect is not considered by the authors. Thus the conclusion of the authors that "the proliferative response of lymphocytes was further emphasized by increased surface hydrophilicity" is not supported by the data.

Authors: Many studies have presented a theoretical and experimental comparison between two approaches to the contact angle measurement, i.e., the drop (water drop in air) and the captive bubble (air bubble in water) methods. In these studies, the general definitions have been shown for the contact angle measured by the drop and captive bubble methods; θ (degree) is the angle between the solid surface and the water drop or the air bubble interface measured inside the drop or bubble. is related to the actual contact angle θ_{ac} by:

$\theta = \theta_{ac}$ for a water drop
$\theta = 180-\theta_{ac}$ for a captive bubble

Thus, in case of the captive bubble method, a higher contact angle (θ) corresponds to an increased hydrophilicity (smaller value of θ_{ac}).