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Original article

Reduction of non-specific adsorption of drugs to plastic containers used in bioassays or analyses

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ABSTRACT

Introduction: Non specific adsorption (NSA) of drugs to plastic or glass containers used in clinical use is well known, but methods for reducing NSA have been rarely reported. We assessed the NSA to various containers and then investigated methods to reduce NSA. **Methods:** Probe drugs (methotrexate, warfarin, chloroquine, propranolol, verapamil, digoxin and paclitaxel) dissolved in water were incubated in conventional or low adsorption containers for 4 h at 4 °C and the NSA was determined by HPLC. They were also dissolved in aqueous methanol or acetonitrile and the NSA to a conventional polypropylene microplate was determined. Finally, tissue culture microplates were coated with silane coupling agents and the effects of the coatings were evaluated. **Results:** Hydrophobic drugs (paclitaxel, verapamil and digoxin) were highly adsorbed to conventional plastic microplates, but in addition to hydrophobic drugs, positively charged drugs were well adsorbed to the tissue culture microplate. Low adsorption microplates could reduce NSA below 15%, but positively charged or neutral hydrophobic drugs showed relatively higher adsorption. Acetonitrile showed stronger NSA inhibition than that of methanol, but the peak shapes of methotrexate and chloroquine were broadened and split. Among the silane coupling agents, GPTMS suppressed the NSA below 10%. Also, AATMS resembled the NSA pattern of GPTMS, but it increased the adsorption of methotrexate to 29%. **Discussion:** On conventional plastic microplates, NSA is mainly driven by hydrophobic interactions, but on tissue culture microplates and low adsorption microplates, in addition to hydrophobic interactions, ionic interactions play a role in the NSA. Therefore, to reduce the NSA to plastic containers, both hydrophobic and ionic interactions should be reduced using amphiphilic organic solvents or neutral and hydrophilic coatings.

1. Introduction

Non specific adsorption (NSA) of drugs to plastic or glass containers used in clinical use is well known, and it decreased the concentration of drugs critically in some cases (Geary, Akood, & Jensen, 1983; Yahya, McElnay, & D'Arcy, 1988).

In bioassays (e.g., plasma protein binding, Caco 2 cell transport experiment, etc.) or analytical conditions, the concentrations of drugs are much lower than in clinical use and, therefore, the effects of NSA on the assays or analyses are considered to be more potent than clinical use (Robert, 2006). For example, 90% or more loss of drug concentration resulted from NSA to ultrafiltration devices and underestimation of drug concentrations during Caco 2 cell transport experiments due to NSA were reported (Joni, Jukka, Timo, Anssi, & Seppo, 2006; Nanying et al., 2002).

NSA is largely the result of binding forces that originate in electromagnetic interactions and, in this case, it is roughly divided into ionic adsorption and hydrophobic adsorption (Di, Li fen, & Jessie, 1995; Joni et al., 2006). Ionic adsorption is an interaction of a hydrophilic group on a surface (e.g., silanol on glass) and ionic groups of drugs (e.g., chloroquine) and hydrophobic adsorption (van der Waals adsorption) is an interaction of a hydrophobic surface (e.g., plastic) and hydrophobic drugs (e.g., paclitaxel, digoxin, verapamil).

Because drug candidates recently became more hydrophobic and less soluble (Christopher, 2000), and the containers used in assays have also started to be made from hydrophobic plastics, hydrophobic adsorption is mainly responsible for NSA (Pradip, 1998). To overcome the NSA to plastic plates, commercially available low adsorption microplates often introduced hydrophilic groups on the surface of the microplates (Rainer, Claudia, Ramona, Günther, & Heinrich, 2008). These modifications reduced the hydrophobic adsorption of highly hydrophobic drugs such as paclitaxel or digoxin (Stan et al., 2004), but the effect on the adsorption of drugs suspected of ionic adsorption remained unclear.

Any method to reduce or eliminate the NSA of drugs which have a wide variety of hydrophobicity and ionization tendencies may be useful. Therefore, we investigated a method to reduce the overall NSA

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to containers used in assays or analytical experiments. The target value of NSA was set to 10%, taking into account the FDA's guidance that allows the maximum deviation on the analysis to be 15% (U.S. Department of Health and Human Services, Food and Drug Administration, 2001).

2. Methods

2.1. Chemicals

Digoxin, methotrexate, propranolol, and (±) verapamil hydrochloride were purchased from Sigma Aldrich (St. Louis, MO, USA). Chloroquine diphosphate, paclitaxel, and warfarin were obtained from Wako Pure Chemical Industries (Osaka, Japan). Tetraethyl orthosilicate (TEOS) was purchased from Sigma Aldrich (St. Louis, MO, USA). Vinyltrimethoxysilane (VTMS), 3 glycidoxypropyltrimethoxysilane (GPTMS) and N 2 (aminoethyl) 3 aminopropyltrimethoxysilane (AATMS) were obtained from Shin Etsu Chemical (Tokyo, Japan).

2.2. Containers

Glass vials and Plate+ were purchased from Tomsic (Tokyo, Japan). Conventional non treated polypropylene microplates, polystyrene microplates, and NoBinding plates were obtained from Greiner Japan (Tokyo, Japan). Multichem™ microplates, Proteosave 96F plates, Enhanced Recovery plates and tissue culture treated polystyrene microplates were provided by GE Healthcare Japan (Tokyo, Japan), Sumitomo Bakelite (Tokyo, Japan), Becton Dickinson (Franklin Lakes, NJ, USA) and Asahi Glass (Tokyo, Japan), respectively.

2.3. Silane coupling agent treatment to microplates

To 49.5 mL of 1% acetic acid, 0.5 mL of a silane coupling agent (TEOS, VTMS, GPTMS or AATMS) was added and stirred for 1 h at room temperature. The hydrolyzed coating agent was added to wells of a tissue culture treated polystyrene microplate and allowed to stand for 1 min at room temperature. After disposal of surplus coating agent, the microplate was dried and condensed at 50 °C for 4 h.

2.4. Evaluation of NSA

Each drug was dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L. To 998 μL of solvent (distilled water, aqueous methanol or aqueous acetonitrile), 2 μL of a probe drug solution was added and stirred. This solution was chilled at 4 °C for 30 min, diluted 10 times by chilled medium (4 °C) in the test container and analyzed sequentially for 4 h using HPLC.

2.5. Analytical methods

The concentration of each drug was measured with a HPLC system (LC 10A, Shimadzu). A reverse phase column (Capcell PAK C18 MG II, 2.0 mm i.d. ×50 mm, Shiseido) was used. The mobile phase consisted of two components: A, 0.08 vol.% trifluoroacetic acid/water; B, acetonitrile. The flow rate was 0.60 mL/min and a linear gradient from 3% B to 70% B for 5 min was used. The temperatures of the autosampler and column were 4 °C and 40 °C respectively, and the injection volume was 40 μL. Detection of each drug was performed *via* UV absorption as follows: 230 nm for propranolol, paclitaxel and verapamil, 243 nm for digoxin, 258 nm for chloroquine, 282 nm for

2.6. Data analysis

The adsorption rate (S) was calculated using the following equation:

$$S = (1 - A_t / A_0) \times 100,$$

where A_0 is the initial peak area, and A_t is the peak area of t in h.

3. Results

3.1. NSA to conventional plastic and glass containers

The probe drugs shown in Table 1 were selected according to their solubility to water, charge in water and adsorption rate to plastic or glass (Bergström, 2003; Brigitte et al., 2000; Elizabeth Kulpinski, 2006; Geary et al., 1983; Hiroki, Kohji, Noriyoshi, Katsuhiko, & Nobuyoshi, 2006; Nanying et al., 2002; Yalkowsky & Dannenfelser, 1992; Zingone & Rubessa, 2005).

To initiate the study, the NSAs of the probe drugs to conventional (non treated) plastic and glass containers were measured. Fig. 1 shows the time course of the adsorption rate of the probe drugs to a conventional polystyrene microplate. The adsorption rates of digoxin, verapamil and paclitaxel exceeded 10%, and the adsorption rate of paclitaxel was the highest among the probe drugs. In this experiment, the adsorption rates of each drug were nearly identical from 2 to 4 h after incubation.

The mean adsorption rates from 2 to 4 h after incubation in the conventional polystyrene microplate, polypropylene microplate and glass vial are shown in Fig. 2. To the conventional plastic microplates, the adsorption rates of paclitaxel were the highest and reached 65 67%, while verapamil and digoxin also adsorbed over 20% (22 42%). In addition to these drugs, propranolol was adsorbed at over 10% to the polypropylene microplate. To the glass vial, chloroquine showed the highest adsorption rate (42%) and the adsorption rate of verapamil was also over 10%.

3.2. NSA to low adsorption microplates

The mean adsorption rates of the probe drugs to low adsorption microplates are summarized in Fig. 3. To a Multichem microplate, the adsorption rate of paclitaxel was the highest (61%) and the adsorption rate of digoxin was over 10%. To a Proteosave 96F microplate, digoxin showed the highest adsorption rate (34%) and the adsorption rates of propranolol, verapamil and paclitaxel were also over 10% (14 18%). To an Enhanced Recovery plate and a NoBinding plate, the adsorption rates of verapamil were over 10% (18 27%), and the adsorption rate of paclitaxel to an Enhanced Recovery plate was slightly over 10%. To a Plate+, paclitaxel showed an adsorption rate over 10% (15%) and the adsorption rates of propranolol and digoxin were slightly over 10%.

Table 1
Physicochemical properties and oral absorption rates of the probe drugs.

Drugs	Solubility in water (mg/mL)	Charge[a]	Absorption (%)
Methotrexate	9.0[b]	Negative	<10[a]
Warfarin	0.019[c]	Negative	<10[a]
Chloroquine	0.67[d]	Positive	70 (Glass)[e]
Propranolol	0.031[f]	Positive	<10[a]
Verapamil	0.0082[f]	Positive	55[a]
Digoxin	0.065[g]	Neutral	60[a]
Paclitaxel	0.00041[h]	Neutral	70[a]

Data are from [a]Nanying et al., 2002, [b]Brigitte et al., 2000, [c]Zingone & Rubessa, 2005, [d]Elizabeth Kulpinski, 2006,[e]Geary et al., 1983,[f]Bergström, 2003,[g]Yalkowsky & Dannenfelser,

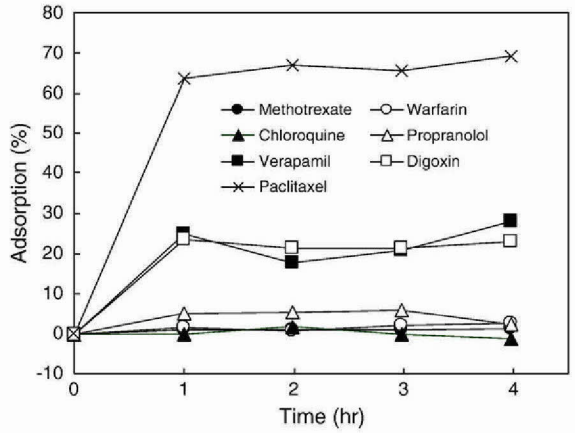

Fig. 1. Time course of the NSA of probe drugs on a conventional polystyrene microplate incubated at 4 °C.

3.3. Effects of amphiphilic organic solvents on the NSA to a polypropylene microplate

The effects of amphiphilic organic solvents on the NSA to a conventional polypropylene microplate were investigated using methanol and acetonitrile as the solvents. The mean adsorption rates of the probe drugs dissolved in various concentrations of aqueous methanol or aqueous acetonitrile are summarized in Figs. 4 and 5, respectively.

The mean adsorption rates of probe drugs decreased inversely with the concentration of methanol or acetonitrile. With 20% methanol, only paclitaxel showed adsorption exceeding 10%, and with 30% or more methanol, the adsorption of all the probe drugs almost disappeared.

In the case of acetonitrile, a reduction in the adsorption was observed at lower concentrations than methanol, the adsorption of all the probe drugs almost disappeared at 20% acetonitrile, but the peak shapes of methotrexate and chloroquine at 20% or higher concentrations of acetonitrile were broadened and split.

3.4. Effects of coatings on the NSA to a tissue culture treated polystyrene microplate

The effects of low adsorption coatings on the NSA to a tissue culture treated polystyrene microplate were investigated using silane coupling agents as the coating agents.

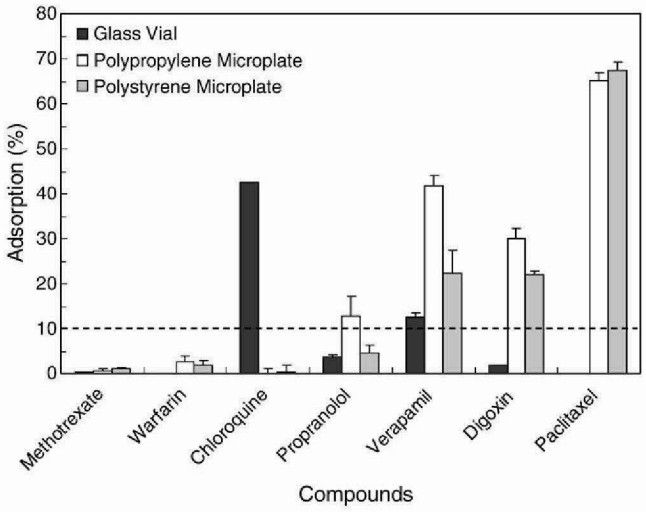

Fig. 2. The NSA to conventional polystyrene and polypropylene microplates and glass vials incubated at 4 °C. The dotted line shows our target value. Each data represents the

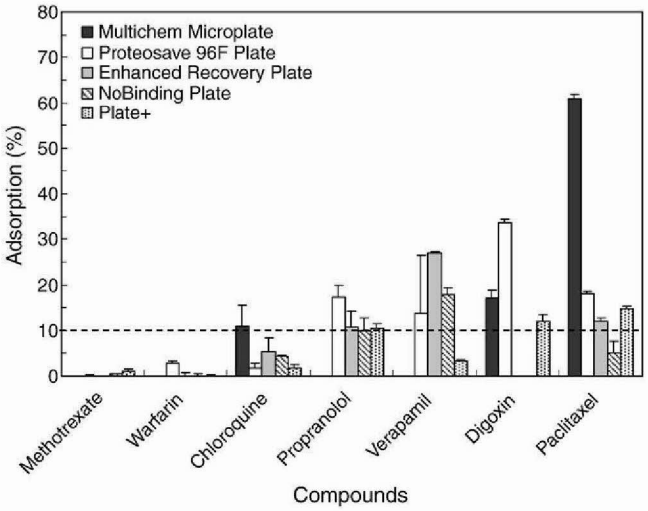

Fig. 3. The NSA to low-adsorption microplates incubated at 4 °C. The dotted line shows our target value. Each data represents the mean ± SD of the adsorption rate from 2 to 4 h after incubation.

The mean adsorption rates of the probe drugs to coated or non coated microplates are summarized in Fig. 6. To the non coated, tissue culture treated polystyrene microplate, verapamil, propranolol, paclitaxel and chloroquine were adsorbed at 65%, 57%, 32% and 23%, respectively. TEOS did not reduce the adsorption of the above drugs to below 10%. VTMS did not reduce the adsorption of verapamil, propranolol or paclitaxel below 10% either, but reduced the adsorption of chloroquine to about 10%. GPTMS effectively suppressed the adsorption of all the probe drugs, and the maximum adsorption rate was 9.6% for paclitaxel. AATMS also effectively suppressed the adsorption of almost all the probe drugs, but increased the adsorption for methotrexate to 31%.

4. Discussion

The surface of plastic microplates commonly seemed to be hydrophobic and the NSA to a microplate was also considered to be mainly mediated by a hydrophobic interaction between the hydrophobic surface of the microplate and also the hydrophobic surface of a compound (Joni et al., 2006). This idea appears to be acceptable in the case of conventional plastic microplates because hydrophobic paclitaxel, verapamil and

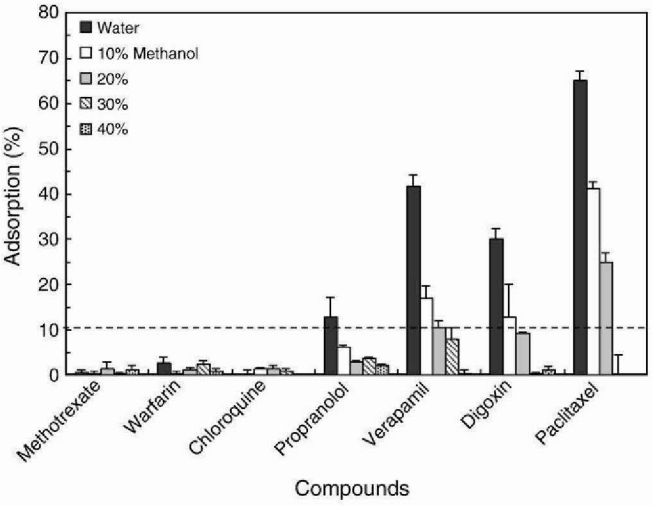

Fig. 4. Effect of methanol on the NSA to a conventional polypropylene microplate incubated at 4 °C. The dotted line shows our target value. Each data represents the

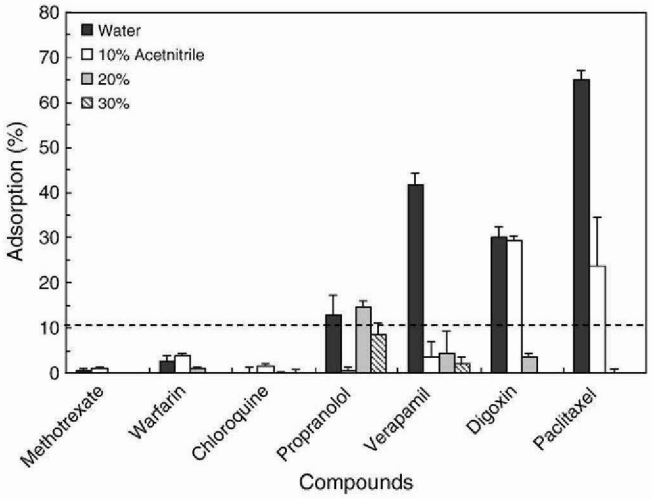

Fig. 5. Effect of acetonitrile on the NSA to a conventional polypropylene microplate incubated at 4 °C. The dotted line shows our target value. Each data represents the mean ± SD of the adsorption rate from 2 to 4 h after incubation.

digoxin showed high adsorption rates to the microplates. However, to tissue culture treated microplates, in addition to the hydrophobic interaction, an ionic interaction may play a role on the NSA because positively charged verapamil and propranolol showed higher adsorption than that of electrically neutral, but more hydrophobic, paclitaxel. The tissue culture treated microplate used in this study was treated with corona which introduced hydrophilic groups like hydroxyl, carbonyl and carboxyl groups to the surface of the plate (Juliana et al., 1997). Therefore, its surface was negatively charged like glass, and adsorbed positively charged drugs. On the other hand, the introduction of hydrophilic groups on the tissue culture microplate by corona treatment was sparse, so the hydrophobic interaction remained and adsorbed hydrophobic drugs.

These tissue culture treatments are used as low adsorption treatments for plastic containers (BD Biosciences, 2002), so the same adsorption characteristics as those of the tissue culture microplate were seen among the low adsorption microplates, except for the Multichem™ microplate, which was made from fluoride containing plastic. The surface of the Multichem™ microplate was hydrophobic like polytetrafluoroethylene and consequently it adsorbed hydrophobic drugs (Yaqi et al., 2003). Other low adsorption

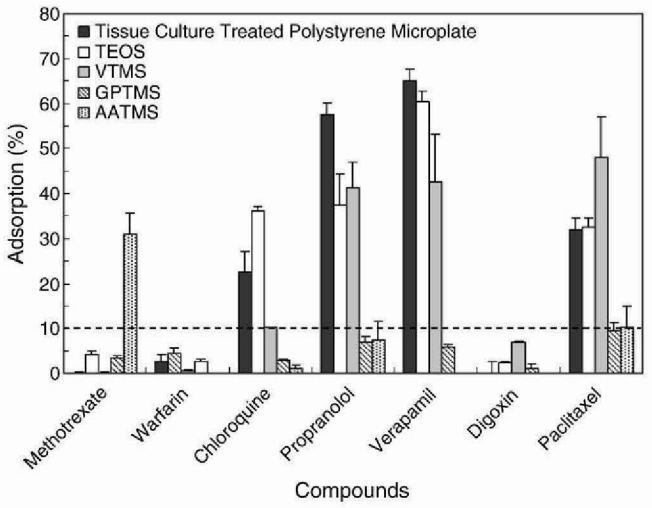

Fig. 6. Effect of low-adsorption coatings on the NSA to a tissue culture-treated microplate incubated at 4 °C. The dotted line shows our target value. Each data represents

microplates have hydrophilic surfaces and showed weak adsorption to neutral and hydrophobic drugs. However, the adsorption of positively charged and hydrophobic verapamil on the Proteosave 96F Plate, Enhanced Recovery Plate and NoBinding Plate which were made from polystyrene, was not reduced compared to that of the conventional polystyrene microplate. The hydrophilic groups introduced on the surfaces of these microplates were negatively charged hydroxyl, carbonyl or carboxyl, so ionic adsorption remained and played a role to adsorb positively charged verapamil.

It was reported that the NSA to a polystyrene plate was reduced if the solvent contained salts (Joni et al., 2006), so with salt solutions, these low adsorption microplates would greatly reduce the NSA. Among these microplates, the NoBinding plate and Plate+ reduced the adsorption of all the probe drugs below 15%. These values were insufficient for our target value, but would be more useful than the others tested.

With amphiphilic organic solvents, the NSAs of all the probe drugs were effectively inhibited from a low concentration (10 20%) of the solvents. The inhibitory effects of acetonitrile on the NSA were stronger than those of methanol, but the retention of drugs to the analytical column was weakened at 20% or higher concentrations of acetonitrile. Therefore, aqueous methanol is a more preferable solvent for new chemical entities of which the physicochemical properties are unknown.

In the above conditions, although aqueous organic solvents were still more hydrophilic than the plastic containers, the NSAs of the probe drugs to the containers were well inhibited. Considering the above results, eliminating the difference in hydrophobicity or hydrophilicity between containers and solvents is not necessary, but rather narrowing the difference is sufficient to reduce the NSA.

Whereas aqueous organic solvents are useful for analytical use, they are limited in cell based assays because of their toxicity (Donna et al., 2008; William, Joseph, & Charles, 1998). Therefore, to reduce the difference in hydrophilicity of the solvents and containers, instead of increasing the hydrophobicity of the solvent, decreasing the hydrophobicity of the container may be viable. To do this, we used some silane coupling agents to coat the surface of the tissue culture treated microplate.

Silane coupling agents are silicon based chemicals that contain two moieties: one is a hydrolysable alkoxy group, such as methoxy, ethoxy, or acetoxy, and another is an organofunctional group, such as amino, methacryloxy, or epoxy, etc. The alkoxy groups of silane coupling agents are hydrolyzed to silanols and they condense with hydroxyl groups on materials. Their silanol groups also react with each other to give a tight siloxane network with their organofunctional groups facing outward, so the surface of the material shows the physicochemical properties derived from the organofunctional groups (Gerald, 1993).

Among the silane coupling agents we used, only VTMS has a hydrophobic organofunctional group, and the surface of the coated microplate repelled water. Its hydrophobicity will enhance the adsorption to neutral and hydrophobic drugs such as paclitaxel and digoxin, whereas it may decrease the ionic interaction to positively charged chloroquine, propranolol and verapamil. TEOS has no organofunctional group and the surface of the coated microplate has free silanol groups. Silanol is ionized in water and the surface of the coated microplate is hydrophilic and negatively charged, so the adsorption of hydrophilic and positively charged chloroquine may be enhanced. Meanwhile, the coating of TEOS seemed to be insufficient to prevent the adsorption of hydrophobic drugs because it lacks an organofunctional group which prevents the adsorption of hydrophobic drugs to the microplate.

GPTMS and AATMS, which have a hydrophilic organofunctional group, inhibited the adsorption of almost all the probe drugs effectively, but only AATMS enhanced the adsorption of a negatively

interaction of the amide group of AATMS and the carboxyl groups of methotrexate. GPTMS also has a hydrophilic glycidoxy group, but the adsorption of positively charged drugs was not enhanced. It was reported that the surface of GPTMS treated glass was electrically neutral and hydrophilic (Rohit, Kenneth, & Arun, 2006), so the surface of a microplate coated with GPTMS will be neutral and hydrophilic, and therefore it reduced the adsorption of all the probe drugs. In a similar fashion to GPTMS, coatings with neutral and hydrophilic groups, such as ether, methacryl and acryl groups would reduce the adsorption of compounds with a wide range of surface properties.

GPTMS could coat a conventional polystyrene microplate, but the reduction in the NSA was insufficient (data not shown). A small amount of a hydroxyl group can be introduced on the surface of a microplate during electron beam sterilization, which is widely used for sterilization of plastic materials, but it seems to be an insufficient scaffold for silanol groups of GPTMS. Therefore, to coat a conventional non treated microplate with silane coupling agents, we should increase the amount of hydroxyl groups on the surface of the microplate. While there are many methods to introduce hydroxyl groups on the surface of plastics, such as UV, electronic beams and atmospheric pressure plasma (Claire, Christelle, Pascal, & Philippe, 2006; Nobuyuki, 2005), UV radiation by a low pressure mercury lamp would be the most convenient method for laboratory use (Halina, Jolanta, Aleksandra, & Alina, 2002).

In summary, we have demonstrated that the NSA is driven by hydrophobic and ionic interactions, and it could be reduced to an acceptable level by reducing these interactions.

On conventional plastic microplates, the NSA is mainly driven by hydrophobic interactions, but on tissue culture microplates and low adsorption microplates, in addition to hydrophobic interactions, ionic interactions play a role in the NSA. Therefore, to reduce the NSA to plastic containers, both hydrophobic and ionic interaction should be reduced using amphiphilic organic solvents or neutral and hydro philic coatings.

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