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Transmembrane transports of acrylamide and bisphenol A and effects on development of zebrafish (*Danio rerio*)

Xun-Chang Fei[a,b], Chao Song[a,1], Hong-Wen Gao[a,*]

[a] *State Key Laboratory of Pollution Control and Resources Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China*
[b] *Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor 48104, USA*

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ABSTRACT

Acrylamide (AA) and bisphenol A (BPA) are two kinds of pollutants with different structures and polarities. AA found in fried and toasted starchy foods can cause developmental and reproductive toxicity and BPA has neuro-, immuno- and developmental toxicities. Their transports in zebrafish (*Danio rerio*) embryos were determined and their toxicity characteristics observed. Approximately 70% of AA was concentrated on the outer membrane surface probably via hydrogen bonds and van der Waals forces, but only 0.3% of AA entered the cytoplasm. In contrast, over 10% of the BPA adsorbed to the cells entered the cytoplasm via the membrane by lipid–water partition. The hydrophilic AA and hydrophobic BPA used different cell transport pathways; AA accumulated on the outer membrane surface whereas BPA readily reached the cytoplasm. AA caused acute and indirect toxicity in developing cells, including serious malnutrition and axial malformation. BPA caused chemical damage to developing cells by causing pericardial edema. The antagonistic effect of the AA/BPA mixture's combinational toxicity to embryos was found and explained by the accumulation of AA on the out surface of membrane inhibiting the transfer of BPA to the cytoplasm.

1. Introduction

Transmembrane transport is the process by which exogenous substances pass through the cell membrane and enter the cytoplasm. Chemicals that cannot bind to a surface receptor must penetrate the cell membrane in order to affect the function of target biomolecules or to cause toxicity. During the transportation process, the chemical must cross the cell membrane, which consists of a phospholipid bilayer and membrane proteins linked to oligosaccharides, and then bind to biomolecules in the cytoplasm before being transported to the nucleus. The mechanisms of toxicity of exogenous chemicals have been elucidated by many studies, which have often focused on characterizing interactions with target molecules [1–5], errors in protein expression [6], alteration of gene sequences [7] and dose–effect relationships [8]. In contrast, transmembrane and intracellular transport of harmful chemicals has received little attention [9].

In the present study, zebrafish (*Danio rerio*) embryos were exposed to two contaminants, hydrophilic acrylamide (AA) which is easily soluble in water with the solubility of more than 2000 g/L at 25 °C and hydrophobic bisphenol A (BPA) which is not easily soluble in water with the solubility of less than 0.3 g/L at 21.5 °C. AA is the precursor of polyacrylamide, which is widely used in the paper, textile and cosmetic industries, and as a soil conditioner and flocculating agent in wastewater treatment [10]. Owing to the wide use of polyacrylamide, AA readily enters drinking water and environmental waters. Studies have shown that AA has neurotoxic [11,12], mutagenic and carcinogenic effects on animals [13–15], and that it causes developmental and reproductive toxicity [16]. Another possible exposure pathway was identified in 2002, as AA was detected in fried and toasted starchy foods such as French fries, potato chips, cereals and bread due to the Maillard reaction in high temperature cooking [17–19]. It can be absorbed into the human body through different routes including the digestive system, and becomes widely distributed among various tissues and organs. Recently, AA pollution has become a focus of attention of many national governments.

BPA is a chemical widely used in the manufacture of epoxy– and polyester–styrene resins and lacquer coatings on food cans [20,21]. The annual production of BPA in the EU increased to 1,150,000 tons in the years 2005–2006 [22]. With the increasing use of products based on BPA, the risk of contamination rises. Reports have described BPA contamination from canned foods

* Corresponding author. Tel.: +86 21 65988598; fax: +86 21 65988598.
E-mail address: hwgao@tongji.edu.cn (H.-W. Gao).
[1] Share the first author.

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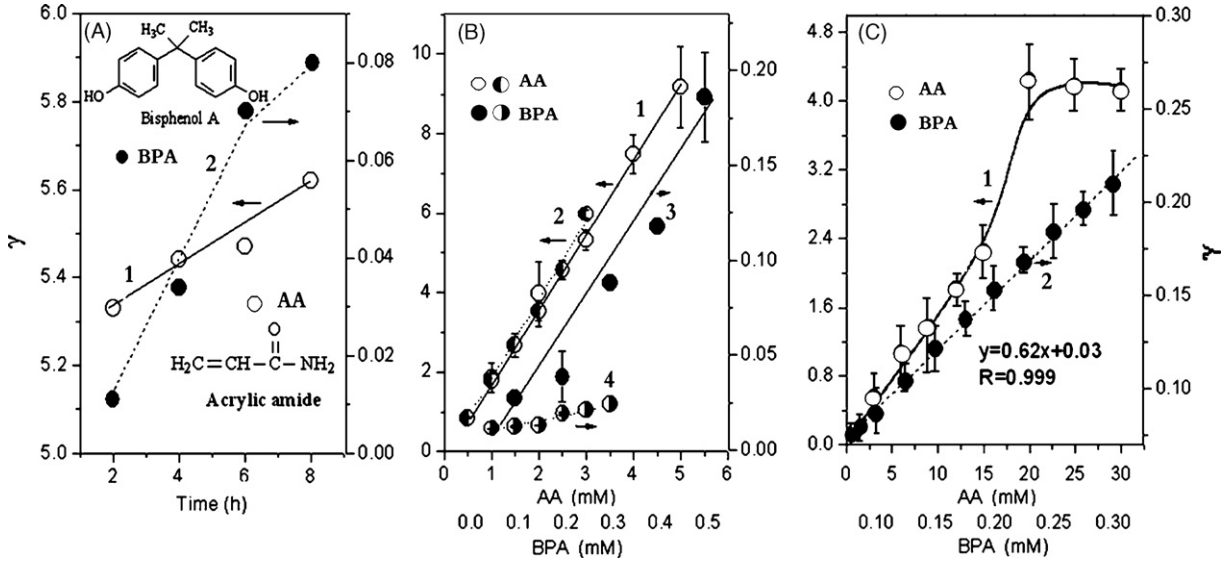

Fig. 1. (A) Effect of exposure time on the amounts of contaminants binding to Zebrafish embryos ($n = 5$): (1) 0.10 mM AA exposure; (2) 0.10 mM BPA exposure. (B) The amount of binding contaminants on embryos ($n = 5$) after the embryos had been incubated with AA, BPA and the AA/BPA mixture for 8 h, respectively: (1) AA with the exposure concentrations: 1.0, 2.0, 3.0, 4.0 and 5.0 mM; (2) AA in the AA/BPA mixture added according to the constant mole ratio, 10 of AA to BPA, i.e. 0.50/0.05, 1.0/0.10, 1.5/0.15, 2.0/0.20 and 2.5/0.25 mM/mM; (3) BPA with the exposure concentrations: 0.10, 0.20, 0.30, 0.40 and 0.50 mM; (4) the same as 2 but BPA in place of AA. (C) The amount of binding contaminants on SMLs (1.00 mg/mL) when the AA or BPA mixed with SML for 2 h: (1) AA from 3.0 to 30 mM and (2) BPA from 0.03 to 0.30 mM.

[23], and as a leachate from landfill waste [24,25]. Humans are exposed to BPA via plastic containers for beverages, and dental patients receiving sealants may ingest it [26]. BPA is estrogenic and is listed as one of the most widespread endocrine-disrupting chemicals (EDCs). Tests on model animals have revealed that it has neuro-, immuno-, developmental and reproductive toxicities [27–30]. It inhibits the steroidogenic cytochrome P45017 (CYP17) [31] and binds protein disulfide isomerase, inhibiting its enzymatic and hormone-binding activities [32,33]. As a potential carcinogen [34,35], it can be converted to DNA-binding metabolites *in vitro* [36]. Absorption of large amounts of BPA through the skin has been shown to cause extensive damage to the liver, kidneys and other organs [37]. It is potentially toxic to embryos and causes genetic defects [38].

Zebrafish (*D. rerio*) have many unique features that make them an important model species for research purposes including a short growth period, high fecundity and spawning transparent eggs [39,40]. The early life stage (ELS) test using zebrafish embryos has been widely used to investigate the acute and chronic toxicity of chemicals that cause significant environmental and human health risks [41–43]. Furthermore, as the zebrafish shares many morphological and physiological characteristics with mammals, toxicological results from studies of zebrafish embryo development can be used to evaluate developmental toxicity in higher vertebrates [44,45]. Therefore, zebrafish embryos are an ideal model for studying vertebrate developmental toxicity, and the ELS test has become an effective method for testing adult fish.

The transport of the contaminants into embryonic cells may be divided into two steps: first, from the extracellular space to the embryonic membrane; second, from the membrane to the cytoplasm. In order to elucidate the correlations among molecular structure, transmembrane transports and toxicological effects of different kinds of pollutants, zebrafish embryos were exposed to AA and BPA, which differ markedly in structure and polarity (Fig. 1A). Interactions of these two different kinds of chemicals with the single membrane liposome (SML) and zebrafish embryos and their transmembrane transport were investigated. The aim of this work is to elucidate the correlation between structure, transmembrane process and toxicological effects of AA and BPA.

2. Materials and methods

2.1. Apparatus and materials

A Model F-4500 fluorospectrophotometer (Hitachi High-Technology Cooperation, Tokyo, Japan) was used for the determination of BPA and a Model Lambda-25 spectrometer (PerkinElmer, Shelton, CT 06484, USA) was used to determine the AA concentration during the process of equilibrium dialysis. An ultrasonic cell disruptor (Model JY92-II, Ningbo Scientz Biotechnology Co., Ltd, China) was used to disrupt the fish embryos. High performance liquid chromatography (HPLC) (Model L-2000, Hitachi, Japan) was used to determine AA and BPA concentrations in three different parts of the embryos (extracellular, membrane and cytoplasm), and was performed using an L-2130 pump, diode array detector (DAD) (Model L-2455) and an inverse-phase column (C_{18}, Model Allsphere ODS-2 5u, 250 mm × 4.6 mm, Alltech Associates, Inc., USA). A freeze-drying instrument (Model K750X, Jintan Etong Electrons, China) was used to prepare lyophilized embryos and a scanning electron microscope (SEM, Model S-4800, Hitachi Inc., Japan) to observe changes in 3D morphology of the embryo surface. An inverted microscope (Model TE2000-U, Nikon Inc., Japan) with a charge-coupled device (CCD) (Evolution^{TM} MP, Media Cybernetics, Japan) and digital photomicrography computer software (Image-Pro *Plus* 6.0) was used to observe the toxicity characteristics of embryos and larvae.

The parental zebrafish were kept in a 25-L tank with the following control settings: 250 mg/L $CaCO_2$ hardness, pH 7.5 ± 0.5, and 10.5 ± 0.5 mg/L dissolved oxygen. The light regime was adjusted to a 14/10 h light/dark cycle at 26 ± 1 °C. The fish were fed regularly with frozen red mosquito larvae from an uncontaminated source. The day before a test was performed, several spawning boxes (12 cm × 20 cm × 12 cm) each containing a mesh (3–4 mm gap) were placed in a tank with 6 male and 3 female fish in each box. Spawning and fertilization took place within 30 min under illumination. The embryos were collected and rinsed with reconstituted medium. Lecithin (Sinopharm Chemical Reagent Co. Ltd., China), 20 mg/mL, was suspended in deionized water and then dispersed ultrasonically at maximal amplitude at 4 °C for 5 cycles of

15 s interspersed with 45 s periods of rest [46]. The lecithin suspension was used to form the SML suspension, and the SML was used to simulate cell membranes *in vitro* and investigate the adsorption of AA and BPA on SML. Carrez I solution was prepared by dissolving 15 g $K_4[Fe(CN)_6]\cdot 3H_2O$ in 100 mL of deionized water and Carrez II solution by dissolving 30 g $ZnSO_4\cdot 7H_2O$ in 100 mL of deionized water [47]. SMLs were co-precipitated only with the Carrez I and II mixture. Phosphate-buffered saline (PBS) at pH 7.0 was prepared by mixing 8 g NaCl, 0.2 g KCl, 3.63 g $Na_2HPO_4\cdot 12H_2O$ and 0.27 g KH_2PO_4 in 1 L of deionized water and was used during cell disruption. A reconstituted medium (ISO 6341-1982) was prepared by mixing 0.294 g $CaCl_2\cdot H_2O$, 0.123 g $MgSO_4\cdot 7H_2O$, 0.065 g $NaHCO_3$ and 0.006 g KCl in 1 L of deionized water and was ventilated close to 100% oxygen saturation with aquarium air-pump (Model ACO-5503, Guangdong Hailea Group, China). It was used in the toxic exposure test as the control solution and all test solutions were prepared with this medium.

2.2. Adsorption of AA and BPA on embryos and SML

From the water-solubility and measurement sensitivity of AA and BPA, their addition concentrations were selected with the constant mole ratio of AA to BPA being 10:1. In the adsorption of AA and BPA on embryos: AA (1.0, 2.0, 3.0, 4.0, and 5.0 mM), BPA (0.10, 0.20, 0.30, 0.40, and 0.50 mM) and the AA/BPA mixture (0.50/0.05, 1.0/0.10, 1.5/0.15, 2.0/0.20, and 2.5/0.25 mM/mM) were added to 5 fish embryos, respectively in 5.0 mL glass tubes and diluted to 3.0 mL with reconstituted medium. Each test was replicated three times consecutively and 225 embryos used in these tests. After incubation for about 8 h, the supernatants were analyzed by HPLC and the free AA and BPA concentrations (c_L) were determined. The mobile phase was 50% deionized water, 50% acetonitrile at a flow rate of 0.8 mL/min; the AA peak appeared at 2.7 min and the BPA peak at 4.4 min. In the adsorption of AA and BPA on SMLs, AA (the concentrations from 3.0 to 30.0 mM) and BPA (the concentrations from 0.03 to 0.30 mM) were mixed with SMLs (1.0 mg/mL) in 10 mL plastic tubes and diluted to 3.0 mL with deionized water, respectively. After mixed for 30 min, 0.15 mL of Carrez I and Carrez II solutions were added. The liquids were mixed thoroughly and then centrifuged for 20 min at 12,000 rpm. The AA and BPA concentrations in the supernatants were determined by HPLC.

2.3. Transmembrane transport n of AA and BPA

Five fish embryos were suspended in 3 mL PBS and ultrasonicated for 10×5 s at 120 w interspersed by 5 s intervals of rest. The suspension was centrifuged for 5 min at 6000 rpm. The cell membrane was separated while the cytoplasm was dispersed in the PBS (Supplementary data Fig. S1). The c_L values of AA and BPA in the supernatants were determined by HPLC and the molar amounts of AA and BPA bound to liposomes, embryo cells (γ) and embryo cytoplasm (γ_{cyto}) were calculated. The γ_{mem} values of AA and BPA remaining on the embryo membranes were calculated by $\gamma_{mem} = \gamma - \gamma_{cyto}$.

2.4. 3D-morphology of the embryo surface

Five embryos were exposed to 4 mM AA or 0.3 mM BPA in glass tubes containing 3.0 mL of deionized water. After incubation for 8 h, the supernatant was removed and the embryos were freeze-dried for 4 h at −55 °C. The lyophilized embryos were observed with SEM and photographs were captured.

2.5. Effects of pH, ionic strength and temperature

Britton-Robinson (BR) buffers at pH (6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) were prepared to examine the pH effect on the interactions of AA or BPA with fish embryo cells. A series of NaCl concentrations were used to examine the effect of electrolyte (0, 50, 100, 150 and 200 mM), and temperature (10, 15, 20, 25, 30, 35 and 40 °C) were set using the thermostat vibrator to examine its effect.

2.6. Tests for toxicity after exposure to AA and BPA

After the embryos were collected and rinsed with reconstituted medium, they were examined with an inverted microscope and the normally developed ones were selected. Two hours post-fertilization (2 hpf) embryos ($n = 15$) and 2 h post-hatching (2 hph) larva ($n = 15$) were mixed with AA or BPA in a 25 mL glass Petri dish, respectively. Stock AA solutions were prepared in reconstituted medium and stock BPA solutions were prepared in dimethylsulfoxide (DMSO). The stock solutions were diluted to the exposure solutions with reconstituted medium. The embryos and larvae in the blank control groups were exposed to the reconstituted medium and in the solvent control groups were exposed to solvent carrier (0.1% DMSO). According to the previous experiment, the 96 h LC50 of AA for embryos is about 70-times higher than that of BPA and that of AA for larvae is about 110-times higher than that of BPA. So, the exposure concentration of AA being approximately 100-fold higher than that of BPA was used in the toxicological exposure test. From an AA stock solution of 7.008 g in 1000 mL of reconstituted medium (100 mM), 25, 12.5, 6.25, 3.125 and 1.56 mM AA were prepared with reconstituted medium. From a BPA stock solution of 27.39 g of BPA in 1000 mL of 0.025% DMSO (120 mM), 0.12, 0.06 and 0.03, 0.015 and 0.0075 mM BPA were prepared with reconstituted medium. Photographs of the toxicity characteristics of the zebrafish embryos and larvae after exposure to AA or BPA were obtained with a microscope camera; the images were compared among control groups in reconstituted medium (A), a test group in 3.125 mM AA (B) and a test group in 0.03 mM BPA (C). The mortality of the zebrafish embryos and larvae was determined in the AA and BPA exposure tests which included the acute toxicity to embryos and larvae of 96 h exposure, and the chronic toxicity of 11 days exposure. The tests were replicated three times consecutively.

3. Results and discussion

3.1. Aggregation of AA and BPA on embryos and SML

In order to investigate the effects of different kinds of pollutants on embryonic development, five embryos were exposed to AA, BPA and the AA and BPA mixture, respectively. The AA and BPA adsorption on developing embryos were exhibited in Fig. 1A. The adsorption of AA approaches an equilibrium after 6 h from curve 1 but that of BPA still has a flat-slope rise from curve 2. From curve 1, 91% of the AA accumulated in the first 2 h and only 9% in the subsequent 6 h. From curve 2, that the adsorption of BPA increased in a linear fashion during the first 6 h. High proportion of AA adsorption in the first 2 h demonstrated that hydrophilic substances can be adsorbed readily onto embryonic cells. In the same time period, lower proportion of BPA adsorption was an evidence for a lower initial binding rate of hydrophobic chemicals to embryonic cells. Therefore, it is possible that BPA kept a dynamic transfer from extracellular liquid to cell, i.e. AA and BPA may exhibit the different cell-transport pathway.

As there was good reproducibility in the mole amounts (γ) of AA and BPA bound to embryonic cells at 8 h across the experiments, so the changes in AA and BPA concentrations within the cells were

determined after 8 h exposure (Fig. 1 B). The γ values for AA (curve 1) and BPA (curve 3) increased with increasing initial exposure concentrations. Linear behaviors of curves 1 and 3 in Fig. 1B represent unsaturated AA and BPA entry into embryonic cytoplasm. In order to study AA and BPA's competitive effect, embryos were incubated in the AA/BPA mixture and the results are shown as curves 2 and 4 in Fig. 1B. Comparing the slope of curves 1 and 2, their slope were close to each other, which indicated that the presence of BPA did not significantly affect the adsorption of AA on to the embryos. However, the slope of curve 4 was significantly smaller than that of curve 3, which showed that the presence of AA significantly reduced the adsorption of BPA on the embryos.

Lecithin is one of the main components of cell membranes, and SMLs prepared by dispersing lecithin in suspension are often used to simulate the phospholipid membrane [9]. *In vitro* experiments examining the interactions between SMLs and AA/BPA demonstrated that the association of AA and BPA with SML approaches equilibrium after 30 min. From the curves presented in Fig. 1C, the γ values of AA and BPA increase with initial concentration, but AA approaches a constant maximum at concentrations exceeding 20.0 mM. In contrast, BPA binding to SMLs increased in a linear fashion with increasing initial concentration from curve 2. There is a constant maximum value for AA adsorption as in Fig. 1C. Therefore, the accumulation of AA on SML corresponds to chemical adsorption. The $-NH_2$ group of AA might bind to polar head groups such as $\rangle C{=}O$ and $\rangle PO_4^-$ in the SML via hydrogen bonds and van der Waals forces. So AA is adsorbed only on the outer surface of the SML. AA adsorption is saturated at 4.5 mol/kg, each lecithin molecule binds 2–3 AA molecules. BPA displays different adsorption behavior due to its hydrophobic aliphatic structure. The observed difference indicates that it was transported across the cell membrane after combined with the hydrophobic binding site of transmembrane protein [48]. The partition of BPA is in agreement with the lipid–water partition law, since the coefficient of BPA was calculated as $K_{lip/w,BPA} = 5260$ L/kg, approaching the reference value [49].

3.2. Transmembrane transport of AA and BPA

The molar distributions of AA and BPA in the extracellular medium, membrane and cytoplasm were determined after exposure of the embryos for 8 h and the results are shown in Fig. 2. From the columns in Fig. 2A, over 70% of the AA is adsorbed on to the cells. More than 99.5% of adsorbed AA bound to the cell membrane, but less than 0.5% is found in the cytoplasm. In Fig. 2B, BPA binding to cells ranged from 10% to 30% with increasing initial concentration. Approximately 10% to 20% of the adsorbed BPA entered the cytoplasm, compared with less than 0.5% for AA. Fig. 2C shows the distributions of AA and BPA when embryos were exposed to the AA and BPA mixture solutions. In the presence of AA, over 96% of the BPA remained free in the medium although molar binding (γ) to the cells increased with increasing initial concentration. Less than 4% of the BPA accumulated on the cells, much less than in the absence of AA when compared to Fig. 2B. However, a maximum of approximately 40% of the BPA bound to the cells entered the cytoplasm (Fig. 2C), which is more than in the absence of AA (Fig. 2B). The transfer constants of AA and BPA, i.e. from membrane to extracellular liquid, were calculated to be $K_{AA} = 0.37$ and $K_{BPA} = 5$ μmol/μmol (Fig. 3A). The cytoplasm–membrane transfer constants were $K'_{AA} = 0.002$ and $K'_{BPA} = 0.12$ μmol/μmol (Fig. 3B). SEM images of the cell membrane surface (Fig. 4) show that exposure to AA caused adhesions among the protuberances and obscuration of the inner membrane layer. There are sinking holes distributed on the membrane after exposure to BPA.

AA distribution between cell membrane and cytoplasm suggests that in addition to the polar carbonyl and PO_4^- heads of the phospholipid layer, AA could bind to polar groups such as the carboxyl groups of cerebrosides and the amide groups of ceramides found in the membrane glycolipid chains, via hydrogen bonds and van der Waals forces. It could explain why more AA molecules bound to the out surface of the cell (Fig. 2A) than on SMLs *in vitro* (Fig. 1C). In contrast to the results of the *in vitro* experiments, AA accumulation on zebrafish embryos failed to reach equilibrium, the amount increased with increasing initial concentration (Fig. 1A).

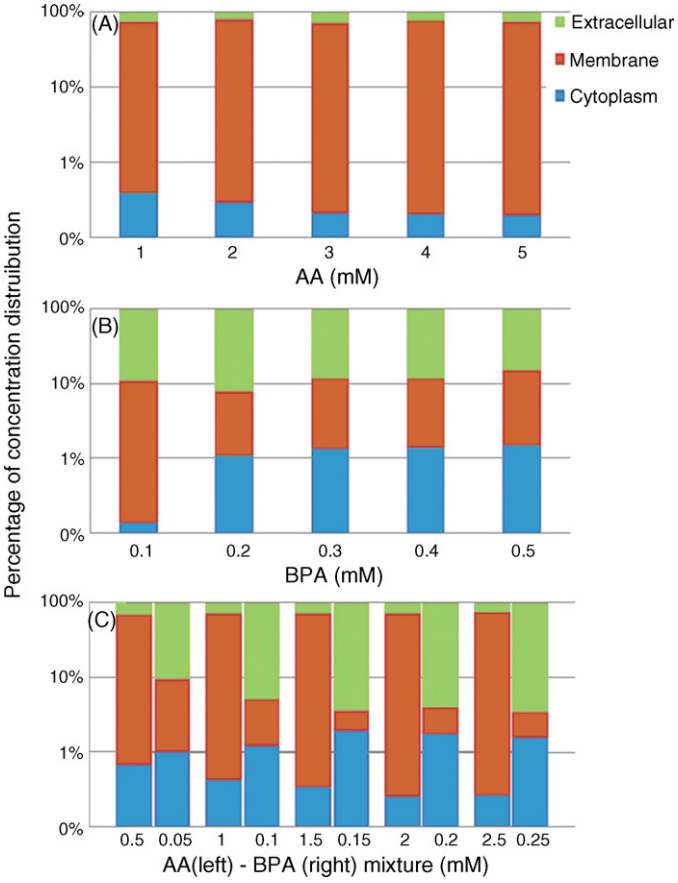

Fig. 2. Distribution of AA and BPA in extracellular fluid, membrane and cytoplasm of embryos ($n = 5$) after incubation in AA, BPA and the AA/BPA mixture for 8 h, respectively: (A) AA exposure with 1.0, 2.0, 3.0, 4.0 and 5.0 mM; (B) BPA exposure with 0.10, 0.20, 0.30, 0.40 and 0.50 mM; (C) exposed to the AA/BPA mixtures contained 0.50/0.05, 1.0/0.10, 1.50/0.15, 2.0/0.20, and 2.50/0.25 mM/mM, respectively.

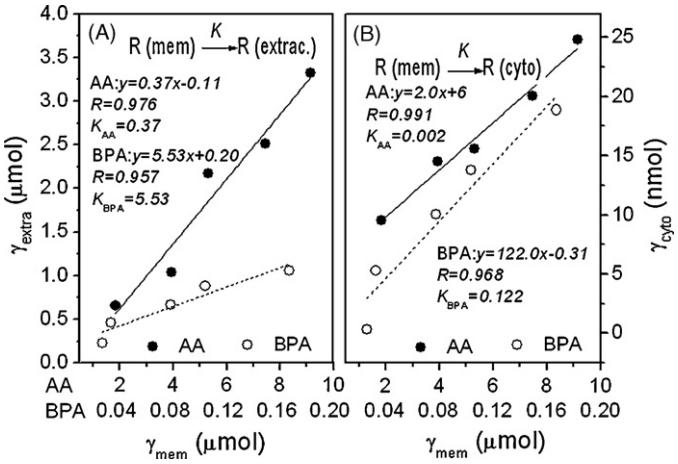

Fig. 3. Plots of γ_{extra} vs. γ_{mem} and γ_{cyto} vs. γ_{mem} for AA (curve 1) and BPA (curve 2), which were calculated from the data in Fig. 2. mem, cell membrane; cyto, cytoplasm; extra, extracellular liquid.

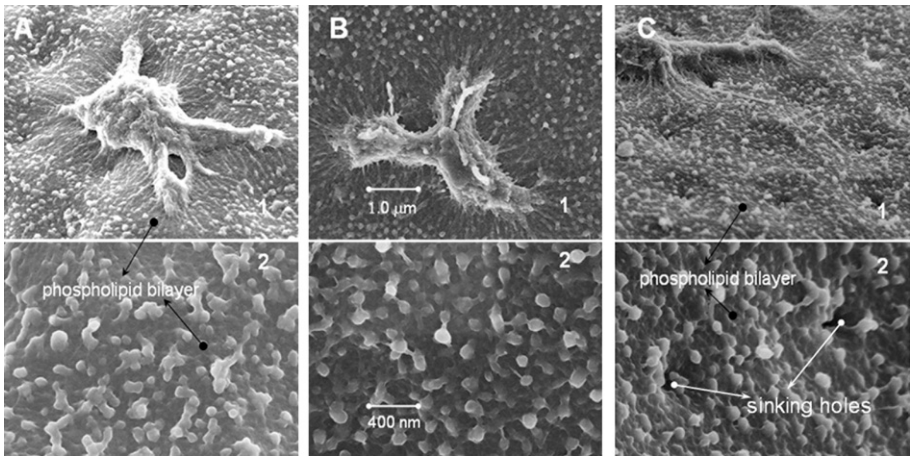

Fig. 4. SEM images of the out surface of zebrafish embryo membrane ($n = 5$) after incubation for 8 h. (A) The control group in the reconstituted medium. (B) 4.0 mM AA exposure group. (C) 0.3 mM BPA exposure group. After exposure, the exposed embryos were freeze-dried at −55 °C for 12 h.

This could be explained by the fact that AA enters the cytoplasm via the membrane protein and rotation of phospholipids during the rapid division of embryonic cells. Values calculated in Fig. 3 indicate that AA accumulates at the surface of the membrane more readily than BPA, while BPA enters the cytoplasm from the membrane much more easily than AA. Hence the observations in Fig. 2 are reassured. Fig. 2C emphasizes the fact that simultaneous presence of AA markedly inhibited BPA transport into the cells. It is in accordance with curve 4 in Fig. 1B. The comparatively much higher BPA concentration in cytoplasm maybe resulted from the association of AA with the outer surface of the membrane, as it could stimulate the flow of the inner phospholipid layer, enhancing the reverse partition of BPA. By comparing the changes in column height in Fig. 2A and C, it is evident that the accumulation of AA in the cell is not affected by the presence of BPA. Therefore, the reasonable conclusion is that the toxicity of BPA to embryo may be lower in the presence of AA, i.e. AA and BPA have antagonistic toxic effects because of the association of AA with the outer cell surface.

Comparison between Fig. 4A and B reveals the dense aggregation of AA molecules on the surface of the outer membrane. Fig. 4C presents a smooth background. Therefore, the barrier-free transport of BPA from the incubation medium to the cytoplasm is confirmed. Sinking holes found in Fig. 4C leads to the conclusion that BPA entered the membrane and caused the softening and dissolution of the phospholipid bilayer.

According to experiment data, derived constant values and direct observations, we hypothesize two complete transfer pathways (*a* and *b*) of AA and BPA as illustrated in Fig. 5. The first step in pathway *a* is the partition of BPA from the incubation medium into the membrane phospholipid bilayer (Fig. 5A). Subsequently, reverse partition of BPA from the membrane to the cytoplasm occurs. This partition and reverse partition both depend on hydrophobic interactions, as described above. Thus, BPA might cause chemical damage, e.g. genotoxicity [50]. In contrast, pathway *b* indicates that AA molecules are adsorbed on to the outer surface of the membrane (Fig. 5B) and may form a compact adhesion shell enclosing the membrane. Very little AA enters the cytoplasm and could be carried towards the developing cells. Unlike BPA, AA is most likely to cause membrane toxicity such as serious reduction of membrane permeability and peristalsis.

3.3. *Effects of pH, ionic strength and temperature on AA/BPA-embryos interactions*

Fig. 6 shows the effects of ionic strength, pH and temperature on the molar binding of AA/BPA to cells. The molar binding of AA decreased with increasing alkalinity and ionic strength (Fig. 6A-1 and B-1). This indicates polar interactions between AA and the cells because the addition of electrolytes would increase the force of repulsion between polar molecules [2], so the molar binding of AA to the cells decreased. The higher temperature is favorable for AA

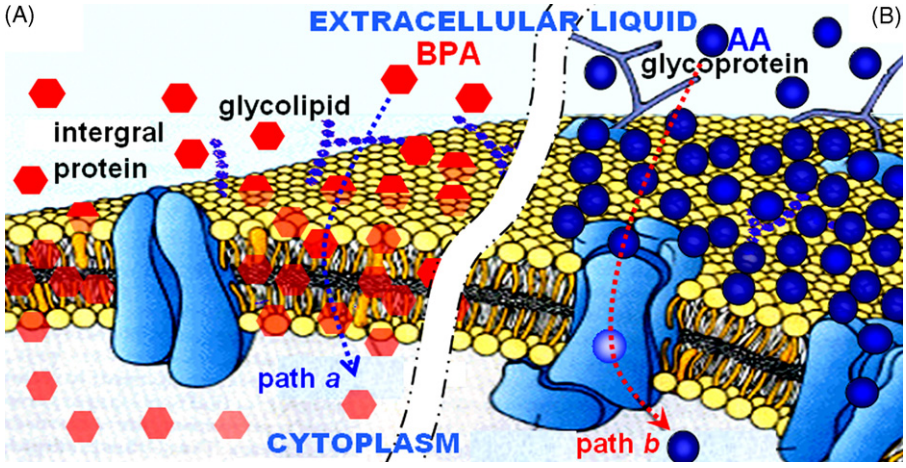

Fig. 5. Cartoon illustration of membrane transport of BPA and AA: Path *a*, possible transport route for BPA (A); path *b*, possible route for AA (B).

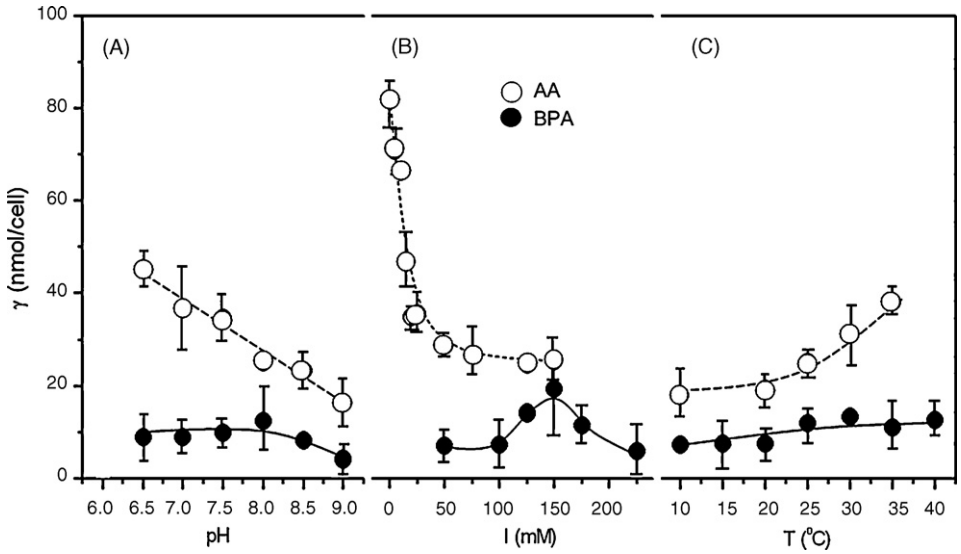

Fig. 6. Effects of pH (A), ionic strength (B) and temperature (C) on the adsorption of AA (0.20 mM; curve 1 -○) and BPA (0.20 mM; curve 2 -●) on embryos (n = 5) with three relicated determinations.

binding to the embryos (Fig. 6C-1). Moreover, the electric bilayer on the cell surface may be damaged by electrolytes. In a weakly alkaline medium, the polarity of the membrane surface rises so that it becomes difficult to concentrate BPA on the membrane (Fig. 6A-2). Increased ionic strength often enhances hydrophobic interactions so that the molar binding of BPA to cells increases and this increase reached a maximum with the increase of ionic strength to 150 mM, and then the molar binding of BPA to cells decreased when the electrolyte concentration exceeded 150 mM (Fig. 6B-2). This may be due to embryo activity weakening in a higher concentration of electrolyte. The molar binding of BPA to the cells is not significantly affected by the pH and temperature (Fig. 6A-2 and C-2). This may be attributed to the fact that the hydrophobic effects were seldom influenced. As a result, low alkalinity and ionic strength, high temperature are favorable for AA binding to the embryos, but the effects of pH, ionic strength and temperature on BPA binding to the embryos are not obvious.

3.4. Effects of AA and BPA on the development of zebrafish embryos and larvae

Fig. 7A shows that the zebrafish embryos hatched at 3 dpf and the hatched larvae developed normally in the control group of reconstituted medium. In the AA exposure groups, the hatching time of the embryos was delayed for 24–48 h (Supplementary data Fig. S2). In addition, pericardial edema (PE), yolk sac edema (YSE), serious axial malformation (AM) and swim bladder deficiency (SBD) were found in the hatched larvae at 7 dpf (Fig. 7B). The hatching time of the embryos was delayed for about 24 h when exposed to BPA (Supplementary data Fig. S2) and PE, YSE and SBD also were found in the hatched larvae at 7 dpf (Fig. 7C). So, there are similarities in the symptoms caused by exposure to AA and BPA, they both cause severe PE, YSE and SBD, but there are some differences in the changes caused by these two chemicals. When embryos are exposed to AA, AM is induced. However, when embryos are exposed to BPA, no AM is induced. Skeletal malformations are lacking after exposure to BPA. Defects occurred in Fig. 7 suggest that normal embryonic development may be destroyed and the immunity of the larvae reduced as AA adsorption may inhibit the uptake of nutrients and other necessary substances. The hatched larvae also presented with spinal curvature, confirming developmental defects (Fig. 7B). Besides, AA that reached the cytoplasm could be transported to the developing cells, causing serious PE and YSE in the hatched larvae. This may be attributed to effects on DNA replication and protein synthesis. BPA caused embryonic dysplasia by affecting the yolk sac (YS), pericardium (P) and cardiovascular system [51,52]. When BPA entered the embryos, it could be transported to the yolk and interact with DNA [53,54], causing serious PE and YSE in the larvae at 7 dpf (Fig. 7C).

From the mortality of the embryos and larvae exposed to AA and BPA (Supplementary data Fig. S3), the absolute lethal dose at 24 h ($LD100_{24\,h}$), i.e. 100% mortality of the embryos just appeared

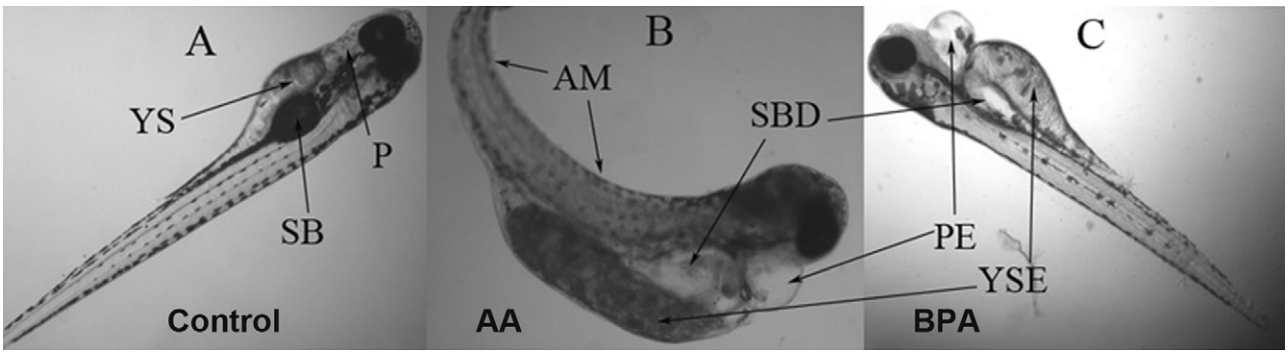

Fig. 7. Microscope photographs of zebrafish larvae in the exposure tests. (A) The control group with reconstituted medium at 7 dpf; (B) 3.125 mM AA exposure group at 7 dpf; (C) 0.030 mM BPA exposure group at 7 dpf. AM, axial malformation; P, pericardium; PE, pericardial edema; SB, swim bladder; SBD, swim bladder deficiency; YS, yolk sac; YSE, yolk sac edema.

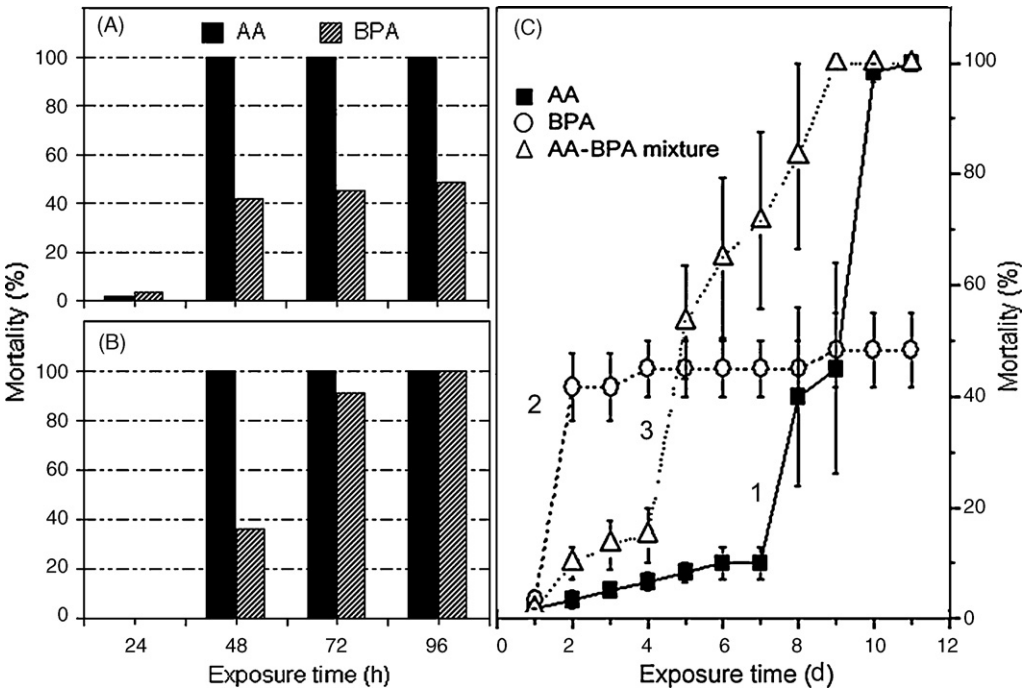

Fig. 8. Mortality of zebrafish embryos and larvae exposed to AA, BPA and the AA/BPA mixture, respectively. (A) Embryos exposure groups with 12.5 mM AA and 0.06 mM BPA, (B) larvae exposure groups with 6.25 mM AA and 0.06 mM BPA, and (C) chronic toxicity exposure for 12 days. The embryos stage: from 1st to 4th day, and the larvae stage after the 4th day. (1) 1.56 mM AA, (2) 0.03 mM BPA and (3) the mixture containing 1.56 mM AA and 0.03 mM BPA.

in 25.0 mM AA and 0.12 mM BPA while that of larvae in 12.5 mM AA and 0.12 mM BPA. The 96 h LC50 of AA (4.28 mM) for embryos is about 70-times higher than that of BPA (0.061 mM) and that of AA (4.20 mM) for larvae is about 110-times higher than that of BPA (0.038 mM). All the embryos died in 6.25 mM AA at 96 h, while only 8% died in 3.12 mM AA at the same time. In contrast, all larvae died in 6.25 mM AA just for 48 h exposure, while no any larvae died in 3.12 mM at the same time. All embryos died in 0.12 mM BPA just for 24 h exposure but just 3% in 0.06 mM BPA at the same time. All larvae died in 0.12 mM BPA just for 24 h exposure but in 0.06 mM BPA for 96 h exposure. Thus, half of $LD100_{24\,h}$ ($HLD100_{24\,h}$) exposed to embryos and larvae can be used to characterize the comparative acute toxicity effect of AA and BPA The $HLD100_{24\,h}$ of AA is 12.5 mM in exposure to embryos and 6.25 mM exposure to larvae and The $HLD100_{24\,h}$ of BPA is 0.06 mM in exposure to embryos and larvae. From Fig. 8A, the mortality of the embryos exposed to $HLD100_{24\,h}$ of AA reached rapidly 100% at 48 h but that to $HLD100_{24\,h}$ of BPA only 42% at 48 h, 45% at 72 h and 48% at 96 h. From Fig. 8B, the mortality of larvae exposed to $HLD100_{24\,h}$ of AA has reached 100% at 48 h but that to $HLD100_{24\,h}$ of BPA only 36% at 48 h, 91% at 72 h and up to 100% at 96 h. Therefore, AA exhibited an obvious acute toxicity effect on the embryos and larvae while BPA a chronic toxicity. The reasons may be that the accumulation of AA on the out surface of membrane blocked the breath passageway and inhibited the exchange of the inner–outer substances of cell, to result in death by suffocation. From Fig. 8C, the combinational toxicity of both AA and BPA to embryos was less than the toxicity of BPA alone (before 4 days exposure), but the toxicity of the AA/BPA mixture exposed to the hatched larvae was higher than that exposed to BPA alone (after 4 days exposure). The antagonistic effect mentioned previously can be explained by the accumulation of AA on the out surface of membrane inhibiting the transfer of BPA to the cytoplasm. After 4 days exposure, the embryos hatched out to larvae and the newly hatched larvae lost the embryo membrane' protection and were exposed to the AA–BPA mixture directly. Furthermore, during embryo development, a serious lack of nutrients and other requirements caused mortality to increase markedly—and this was a synergistic effect.

4. Conclusions

Zebrafish embryos were exposed to AA and BPA, two chemicals with differently structure and polarity. Hydrophilic AA and hydrophobic BPA showed different interactions with the embryos and different distributions in different parts of the embryos. After 8 h exposure, approximately 70% of AA accumulated on the outer membrane surface enclosing the embryo and only 0.3% entered cytoplasm, but over 10% of the BPA adsorbed onto the embryos entered the cytoplasm by lipid–water partition. AA and BPA have different transmembrane distributions and showed different transmembrane pathways, this may cause different toxicity characteristics. AA mainly caused physical damage of embryo membrane, e.g. accumulation on the out surface of the embryonic membrane and leading to serious AM, but BPA transfer to the developing embryo, e.g. causing serious deformities in the cardiovascular system, i.e. serious PE and YSE. When embryos were exposed to both AA and BPA, the association of AA on the out surface of the membrane inhibited the BPA enter the embryos, so that their combined toxicity appeared antagonistic during the embryos stage, but not the larvae stage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.08.007.

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