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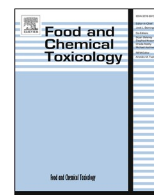

Comparison of the developmental effects of lactase or bisphenol A antibody immobilized polycaprolactone/silk fibroin nanofibers on zebrafish embryos

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A R T I C L E I N F O

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A B S T R A C T

This study aimed to detect the biocompatibility of bioactivated polycaprolactone/silk fibroin-based nanofibers *in vivo* using zebrafish embryos. Anti-Bisphenol A (BPA) antibody or lactase enzyme was immobilized on electrospun nanofibers, for making the nanofiber bioactive. Lactase immobilized nanofiber was developed to hydrolyze lactose and produce milk with reduced lactose. Anti-BPA antibody immobilized nanofiber was developed to remove bisphenol A from liquids. To test the biocompatibility of the bioactive nanofibers, the zebrafish embryos were divided into 4 groups; control, raw nanofiber, lactase immobilized nanofiber, and anti-BPAantibody immobilized nanofiber groups. In nanofiber-based exposure groups; nanofibers were incubated separately in the embryonic development medium. Subsequently, the embryos were kept in these development mediums for 72 h post-fertilization (72 hpf) and their developmental analyzes were performed. At the end of 72 hpf, zebrafish embryos were homogenized. Lipid peroxidation and nitrite oxide levels, and superoxide dismutase and glutathione-S-transferase activities were determined to monitor the disturbance of oxidant-antioxidant balance in zebrafish embryos. Exposure to bioactive nanofibers slightly disrupted the oxidant-antioxidant balance, but this change did not affect the mortality and hatching times of the embryos. In conclusion, zebrafish embryos have been effectively used in biocompatibility testing for bioactive nanofibers suggesting that these materials are biocompatible.

1. Introduction

The immobilization of enzymes and antibodies on nanofibers has potential applications in biotechnology and food science. Bioactivity can be imparted to nanofibers by enzyme and antibody immobilization. The immobilization process can improve the stabilization of the immobilized enzyme or antibody as well as their specific use, but toxicological evaluation of such bioactive materials is also required to ensure their safe use in food and beverage applications. (Asal et al., 2019; Marques et al., 2020).

In this study, PCL/SF based nanofibers were bioactivated by the immobilization of the lactase enzyme and anti-BPA antibody. The polycaprolactone (PCL) and silk fibroin-based nanofiber matrix was prepared using the electrospinning method. PCL is frequently used in the production of biomedical materials because it has high biocompatibility and is biodegradable (Woodruff and Hutmacher, 2010). Silk fibroin, the second polymer of the hybrid nanofiber used in this study, is a protein in the fiber structure and has biocompatibility, and superior material properties. Silk fibroin is used as a biomedical material in cell culture, wound dressing and drug release studies, as a support material in enzyme immobilization and as a skeleton in bone cell engineering (Hardy et al., 2008; Vollrath and Porter, 2009). Lactase enzyme and anti-BPA antibodies were immobilized on PCL/SF nanofibers using physical and chemical adsorption methods, respectively. While the physical adsorption strategy provides an easy incubation step, the chemical adsorption methodology requires the use of a variety of binding agents.

Lactase enzyme immobilized nanofiber was used to reduce the lactose amount in dairy products (Yılmaz-Karaoğlu et al., 2022) and nanofibers immobilized with anti-BPA antibody were used to remove BPA from liquids.

Regardless of the functional, chemical or physical properties, biomaterials need to be thoroughly investigated before use (Liu et al., 2020). These materials should not be carcinogenic, should not carry

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toxicological risks and should not adversely affect the development or reproduction of the human body (Jedlowski et al., 2019). Traditional test methods for the detection of the toxicity of new products have relied mainly on *in vitro* investigations with ex vivo tissue specimens or cell cultures, as well as *in vivo* experimental animal models such as rats (Lee and Lin, 2022; Myers et al., 2017).I n this study, the biocompatibility of bioactivated PCL/SF based nanofibers were tested using zebrafish (*Danio rerio)* embryos, a model organism known for being sensitive to toxic chemicals. Zebrafish and zebrafish embryos have been frequently preferred in many toxicology-based studies because of their similarities with human cell metabolism (Uren-Webster et al., 2010). They can also serve real-time monitoring of toxicological events and a their consequences on embryonic development. Using zebrafish embryos for biocompatibility tests is consistent with the ISO 10993 guideline document that also permits the use of the zebrafish embryo toxicity (ZET) test to evaluate the safety of polymeric compounds (Sharma and Luthra, 2023). The use of zebrafish embryos also reduce risks and costs, and eliminate the requirement for animal testing (Cassar et al., 2020; Chahardehi et al., 2020; Pereira et al., 2019; Zielińska et al., 2020).

2. Material and methods

2.1. Preparation of PCL/SF-based nanofiber

20 mL of a dimethylformamide and dichloromethane (1:4 v/v) solution containing 2g silk fibroin (Shanghai Soyoung Biotechnology Inc., China) and equal amount of PCL (Corbion Purac, Netherlands) were mixed with magnetic stirer at room temperature for 24 h (h). Then electrospun on to the breast milk bag (Inovenso NE200, Turkey) for 3 h under optimal conditions (Gurel-Gokmen et al., 2021).

2.2. Anti-bisphenol A antibody immobilization on PCL/SF-based nanofiber

Anti-Bisphenol A antibody (20 μg/mL, ACRIS, Germany) was immobilized onto the PCL/SF nanofiber (3×3 cm^2) by the method of Garcinuno et al. (2000).

2.3. Lactase enzyme immobilization on PCL/SF-based nanofiber

Lactase (Mayalact 5000 AC, Mayasan Turkey) enzyme from *Bifidobacterium bifidum* is used in the production of lactose-free fermented dairy products. 5000 NLU (neutral lactase unit) lactase enzyme was spread onto the PCL/SF-based nanofibers (3×3 cm^2) and incubated at 37^{0}C for 2 h. Then activated PCL/SF nanofiber was washed with phosphate buffer (0.02 M, pH 6.5) Lactase was immobilized onto the PCL/SF-based nanofiber by the physical adsorption method (Yılmaz-Karaoğlu et al., 2022).

2.4. Zebrafish Care and feeding procedure

The studies with zebrafish embryos were carried out in the Zebrafish Research Laboratory of Marmara University, Dentistry Faculty. As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC. Zebrafish were housed in a computer-controlled ZEBTEC (United Kingdom) aquarium. Circadian rhythms were created by installing a lighting system with 14 h of daylight and 10 h of darkness per day. The temperature was adjusted to.

28 ± 1 °C and humidity and 61% and kept constant with the automation system. The water pH of the system was adjusted between 6.9 and 7.2 and kept constant. With the filtration system, which includes physical and u.v. systems, the environment of the fish is cleaned. They were fed twice a day with dry food and once with live food, a total of three times. Embryos were collected daily from the tanks. Embryonic developmental stages were monitored under the Zeiss Stereo Discovery V8 microscope (Karaman et al., 2020a).

2.5. Zebrafish embryos

Male and female zebrafish were transferred to rearing tanks at a ratio of 2:1 before dark. Mating, spawning, and fertilization occurred as soon as 30 min after the onset of morning light. Fertilized embryos fell into compartments located under the mating tanks. Embryos that were fertilized and reached the appropriate developmental level were selected under a stereo microscope, separated from the unfertilized ones, and collected. Then selected embryos were kept at 28^{0}C in an zebrafish embryos were maintained in E3 embryo medium throughout the experiment (Bhasin et al., 2016). E3 medium is the standard hatchery solution for zebrafish embryos containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_{2,}$ 0.33 mM $MgSO_4$, and 0.1 % methylene blue (Cunliffe, 2003).

2.6. Experimental groups

Zebrafish embryos were divided into four groups; control, raw nanofiber (unactivated), lactase enzyme immobilized nanofiber, and anti-bisphenol A (BPA) antibody immobilized nanofiber.

No treatment was applied to the embryos in the control group.

Raw nanofiber group; consisted of embryos exposed to untreated PCL/SF-based nanofiber incubated in a zebrafish embryo development medium for 1 or 10 h.

Lactase enzyme immobilized nanofiber group; consisted of embryos exposed to PCL/SF-based nanofibers immobilized with lactase enzyme incubated in a zebrafish embryo development medium for 10 h.

Anti-bisphenol A antibody immobilized nanofiber group; consisted of embryos exposed to PCL/SF based-nanofibers immobilized with anti-bisphenol A antibody incubated in a zebrafish embryo development medium for 1 h. After incubation for the specified periods, the nanofibers were removed from the embryo development medium.

2.7. Embryo morphological and developmental analyzes

100 embryos of each groups were separated into 5-embryo subgroups and each sub-group embryos were placed in 20 mL E3 mediums treated with nanofibers and monitored for 72 h post fertilization (hpf). Therefore, the tests were repeated 5 times with 20 embryos in each. Indicators of development including yolk sac, anal pore, pectoral fin, and swim bladder were used for embryo staging (Westerfield, 2000).

The embryos' mortality and hatching (embryo's complete emergence from the chorion) rates were recorded at 72 hpf. Immobile embryos that did not respond to physical stimulation were considered dead and removed from the petri dishes. At the end of 72 hpf embryos of all groups were homogenized and were stored at −20 °C.

2.8. Biochemical analyzes

For the biochemical analysis a total of 5 biological replicates of pools of zebrafish embryos at 72 hpf (20 embryos/pool; 5 biological replicates for each group) were used. Embryos were homogenized in 1 mililiter of PBS per pool, and a fast centrifugation (1878×*g* for 10 min at 4 °C) was performed. The supernatant was used for the analysis of the total protein, lipid peroxidation (LPO), nitric (NO) levels, as well as superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities.

2.9. Total protein determination

The total protein level was determined by the method of Lowry et al. (1951). Total protein level was expressed as mg/dL and used to present the results of the parameters per protein.

2.10. Lipid peroxidation determination

Malondialdehyde (MDA) level, an end product of lipid peroxidation (LPO), as a thiobarbituric acid reactive substance was determined by the method of Ledwozyw (Ledwożyw et al., 1986). Zebrafish embryo homogenate (0,25 mL) was mixed with trichloroacetic acid solution (1.22 M, 0.6 M HCl). 15 min later, the mixture was incubated in a 0,75 mL solution of thiobarbituric acid (0.047 M) in a boiling water bath for 30 min. After adding 2 mL of n-Butanol, the mixture was centrifuged at 1878×*g* for 10 min. The absorbance rates of butanol phase were measured at 532 nm. LPO was presented as nmol MDA/mg protein.

2.11. Nitric oxide determination

Nitric oxide (NO) was determined by the method of Miranda et al. This method is based on the reduction of nitrate to nitrite by vanadium (III) chloride (VCl_3) (Miranda et al., 2001). The zebrafish embryo homogenates were centrifuged at 1878×*g* for 10 min 0.3 mL supernatant was mixed with the equal amount of 0.3 M NaOH and incubated for 5 min. Then 0.3 mL of 10% $ZnSO_4$ was added to this mixture and centrifuged at 23.000×*g* at +4 C° for 5 min 0.3 mL VCl3 (0.05 M) was mixed with the deproteinized zebrafish embryo homogenizate. 0.15 mL of N-(1-Naphthyl)-ethylenediamine dihydrochloride (0,1M) and 0.15 mL of sulfuric acid (5%) were added and incubated 30 min at 37 °C. The absorbance was measured at 540 nm. NO concentrations were presented as nmol NO/mg protein.

2.12. Superoxide dismutase (SOD) and glutathione-S-transferase (GST) activity determination

SOD activity was determined using the potential of riboflavin-sensitized o-dianisidine to increase the rate of photooxidation (Mylroie et al., 1986). PBS (pH 7.8), o-dianisidine dihydrochloride, and riboflavin are mixed. The reaction was initiated by adding riboflavin to the mixture. The absorbance of the mixture was measured at 460 nm, and the data were presented as the quantity of enzyme units per gram of protein.

GST activity was measured according to the method of Habig et al. (Habig and Jakoby, 1981). 1,5 mL PBS (0.1 M, pH 6.5) was mixed to the 0.5 mL of zebrafish embryo homogenate. Then 50 μL GSH (60 mM) and 50 μL 1-chloro-2,4-dinitrobenzene (CDNB, 60 mM) were added to the mixture. The absorbance differences were measured at 340 nm for 3 min.

2.13. Statistical analysis

GraphPad Prism 6.0 package program (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyzes. The normality of the data was checked using the Shapiro-Wilk test before applying parametric tests. The one-way ANOVA followed by Tukey's post hoc test was carried out for the evaluation of the data. The results were presented as the mean and standard deviation (SD). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of PCL-silk fibroin nanofiber

When the morphology of the PCL/SF-based nanofiber was examined, there were no beads in the nanofiber structure and the distribution was homogeneous (Fig. 1).

The diameter of the thinnest fiber was determined approximately 54.69 nm and the diameter of the thickest fiber was determined approximately 379.9 nm in the SEM analysis (Fig. 1). The mean fiber diameter of the PCL/SF-based nanofiber was determined as approximately 208 nm by calculating the average of the measured diameters of fibers from Fig. 1.

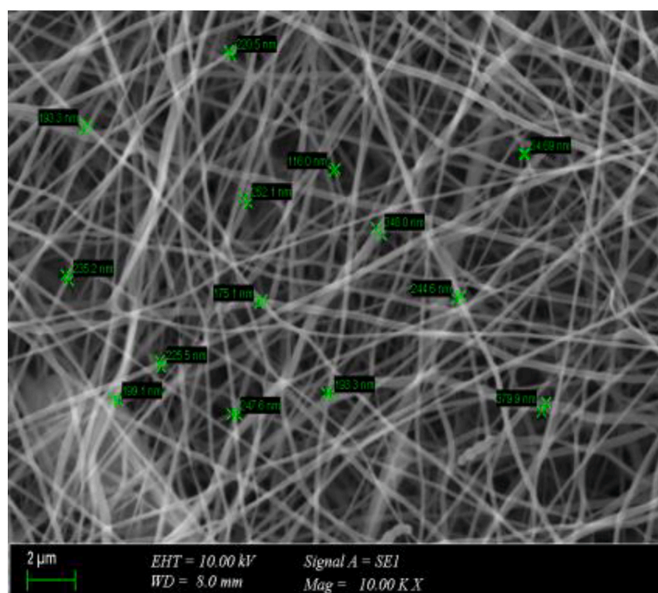

Fig. 1. SEM image of polycaprolactone/silk fibroin-based nanofiber (x10000).

3.2. The determination of the incubation time of antibody or enzyme immobilized nanofiber in zebrafish embryo development medium

The incubation time of nanofibers containing anti-BPA antibody in zebrafish embryo development medium was determined as 1 h. When the anti-BPA antibody immobilized nanofibers were kept in an embryo development medium for 10 h, mortality rate percentages were determined as 100 % at the end of 24 hpf. When the anti-BPA antibody immobilized nanofiber was kept in embryo development medium for 1 h, there was no statistically significant change in mortality rate percentages at 24, 48, and 72 hpf compared to the control group. Since the lactase-immobilized nanofiber had to be kept at +4 °C overnight to prepare lactose-reduced milk, 10 h of incubation was applied to the nanofibers immobilized with lactase in the E3 medium. Incubation of the lactase-immobilized nanofiber in an embryo development medium for 10 h did not cause a significant change in the mortality rate percentages at 24, 48, and 72 hpf compared to the control group.

3.3. Results of zebrafish embryo morphological and developmental analyzes

The mortality rates of the control group and nanofiber-based exposure groups were presented in Fig. 2. No significant differences were observed between the percentages of the mortality rate of raw nanofiber, lactase enzyme immobilized nanofiber, and anti-BPA antibody immobilized nanofiber groups compared to the control group at 72 hpf (Fig. 2).

Fig. 3 shows the hatching rates control group and nanofiber-based exposure groups at 72 hpf. Accordingly, the hatching rate in the control group at 72 hpf was found to be 100%. At 72 hpf, there was no statistically significant difference in hatching rates between the nanofiber-based exposure groups compared to the control group.

Representative images of zebrafish embryos at 24, 48, and 72 hpf in the control group and nanofiber-based exposure groups are shown in Fig. 4. No developmental abnormalities and visible malformations were observed in the control group and the nanofiber-based exposure groups at 24, 48, and 72 hpf.

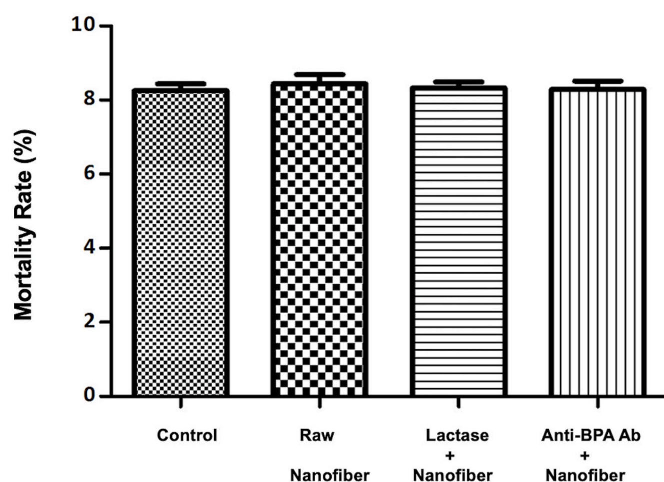

Fig. 2. Mortality rate of zebrafish embryos at 72 h post-fertilization **BPA:** Bisphenol A, **Ab:** Antibody.

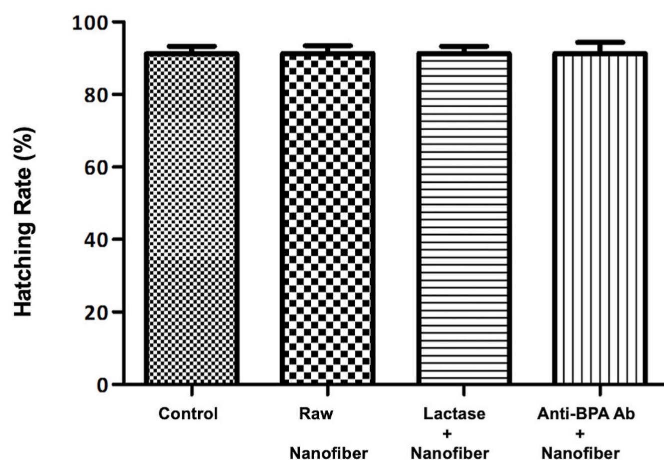

Fig. 3. Hatching rate of zebrafish embryos at 72 h post-fertilization **BPA:** Bisphenol A, **Ab:** Antibody.

3.4. Results of biochemical analyzes

3.4.1. Comparison of total protein levels

No significant differences were found in the total protein level between the control group and nanofiber-based exposure groups.

3.4.2. Comparison of lipid peroxidation levels

Lipid peroxidation was determined by measuring the MDA level. Accordingly, when the MDA levels of the control group and nanofiber-based exposure groups were compared, the MDA levels of the lactase enzyme-immobilized nanofiber group and the anti-BPA antibody immobilized nanofiber group were significantly higher than the control group. No statistically significant difference was found in the MDA levels of the raw nanofiber group compared to the control group (Fig. 5).

3.4.3. Comparison of NO levels

NO levels significantly decreased in the raw nanofiber, lactase enzyme-immobilized nanofiber and the anti-BPA antibody-immobilized nanofiber groups compared to the control group (Fig. 5).

3.4.4. Comparison of SOD activities

SOD activities of the lactase enzyme immobilized nanofiber and the anti-BPA antibody immobilized nanofiber groups were found to be decreased compared to the control group. There were no significant differences between the lactase enzyme-immobilized nanofiber and the BPA antibody-immobilized nanofiber exposure groups (Fig. 5).

3.4.5. Comparison of GST activities

GST activities of the lactase enzyme immobilized nanofiber group and the BPA antibody immobilized nanofiber groups were found to be decreased compared to the control and raw nanofiber exposure groups. There were no significant differences in the GST activities of the lactase enzyme-immobilized nanofiber and the BPA antibody-immobilized nanofiber exposure groups (Fig. 5).

4. Discussion

Recent studies have provided extensive opportunities for new applications in the biocompatibility testing of different chemicals and biomaterials (Hayes and Loomis, 1996; Karaman et al., 2020b). The problem is due to the absence of optimized *in vivo* methods for testing

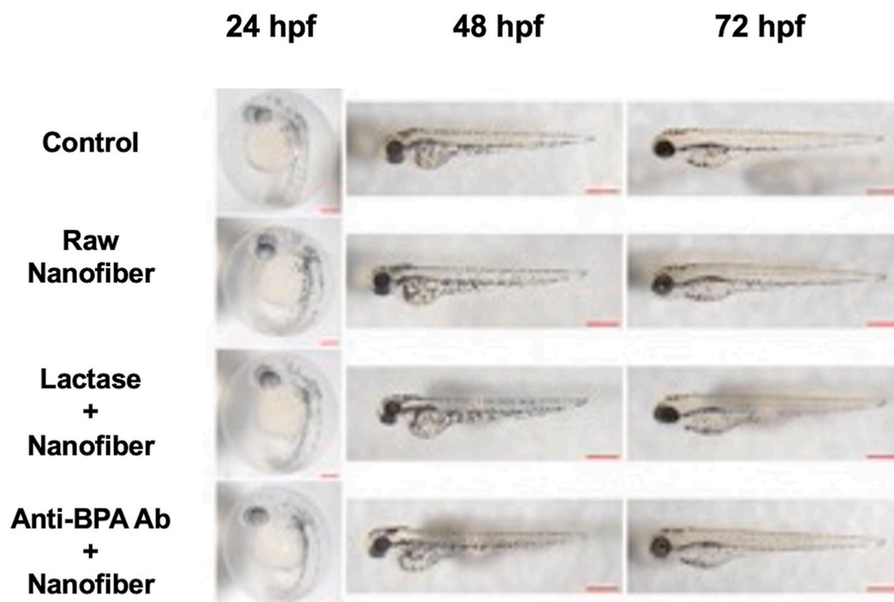

Fig. 4. Images of embryos in the control group and nanofiber-based exposure groups at 24, 48 and 72 hpf. **BPA:** Bisphenol A, **Ab:** Antibody.

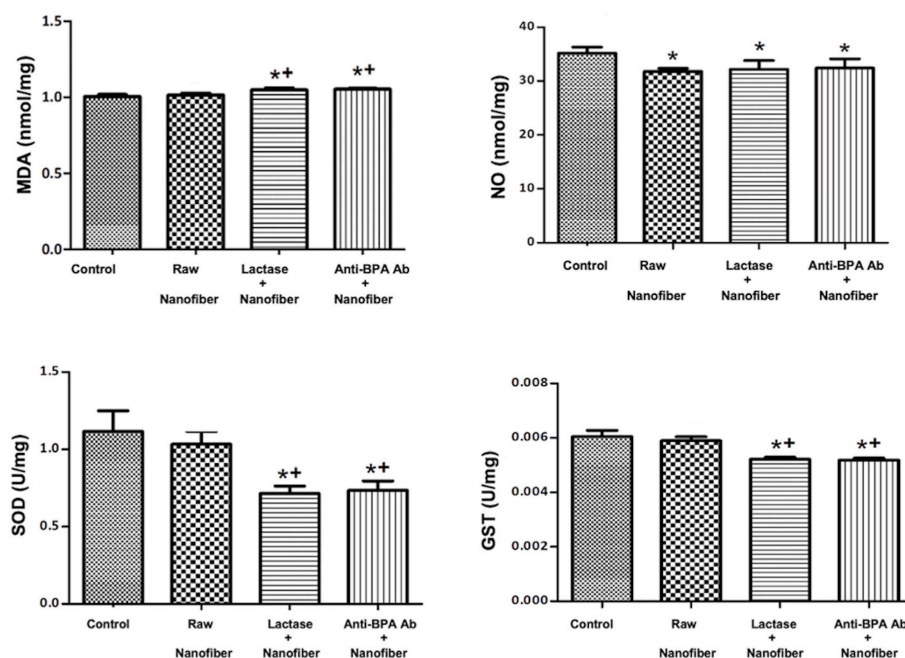

Fig. 5. MDA and NO levels, SOD and GST activities of zebrafish embryos **MDA:** Malondialdehyde, **NO:** Nitric oxide, **SOD:** Superoxide dismutase, **GST:** Glutathione -S-Transferase, **BPA:** Bisphenol A, **Ab:** Antibody *: $p < 0.05$ compared to control group +: $p < 0.05$ compared to the raw nanofiber group.

nanofiber-based biomaterials on zebrafish embryos. In this study, biocompatibility testing was developed using lactase enzyme and anti-bisphenol A antibody immobilized PCL/SF based nanofibers as a model. In our previous studies, it has been demonstrated that lactase enzyme and anti BPA-antibody immobilized nanofibers both perform their intended functions (Gurel-Gokmen et al., 2021; Yılmaz-Karaoğlu et al., 2022). Although PCL and silk fiber were recognized to be biocompatible materials at the beginning of this study, a raw nanofiber group was included to the experimental groups as the effects of the solvent were unknown. In the raw nanofiber group, nanofibers without enzyme or antibody were incubated in the E3 zebrafish embryo development medium. The incubation of raw nanofiber in the development medium did not affect the hatching and mortality rates of the zebrafish embryos. Therefore, any toxicity detected was thought to be related to the immobilization method or the immobilized enzyme/antibodies. In this study, dimetilsuberimidate was used as a cross-linking agent for the immobilization of the anti-bisphenol A antibody. Ten hours of incubation of the antibody-immobilized nanofiber in the embryo development medium showed a toxic effect on zebrafish embryos while lactase-immobilized- nanofiber did not cause any toxic effect on zebrafish embryos within 10 h. Although it was not measured, this result may be associated with the release of the dimethylsuberimidate from the antibody-immobilized nanofiber to the embryo development medium. When the anti-BPA antibody immobilized nanofiber was incubated for 1 h, it did not cause any toxic effect on the zebrafish embryos. This finding showed that no chemicals were released into the embryo development environment within 1 h. Matos et al. showed that the toxic effects of ZnS and CdS in zebrafish are associated with an increased rate of accumulation with increasing concentration (Matos et al., 2020). In the study of Wang et al., cationic poly(amidoamine) dendrimers containing surface amino groups in zebrafish embryos result in 100% death after 24 h of incubation. They stated that dendrimers with cationic amine groups can be more toxic than neutral poly(amidoamine) dendrimers with amino acid ethanol surface groups and anionic dendrimers with succinamic acid end groups. (Wang et al., 2020). In this study, there was no statistically significant time-dependent change in the raw nanofiber-exposed group's mortality rates at 24, 48, and 72 hpf.

The anti-BPA antibody immobilized nanofiber caused 100% mortality in embryos at 24 hpf. When this bioactivated nanofiber incubated in the embryo development medium for an hour, it did not exhibit any toxic effects. This finding demonstrates that the anti-BPA antibody immobilized nanofiber is appropriate for 1 h of contact and has no adverse effects during that period.

Ten hours incubation of lactase immobilized nanofiber did not cause any mortality at 24, 48, and 72 hpf. Therefore, it can be said that the safe contact time of the lactase-immobilized nanofiber was determined as 10 h or less.

Zebrafish embryo development stages were monitored by modifying the existing methods that investigate the possible toxic effects of chemicals or environmental pollutants. (Ateş et al., 2018; Üstündağ et al., 2017). In studies on the toxic effects of drugs, different concentrations of drugs are added to the zebrafish embryo development medium and the lethal dose of the drug is determined. Whereas in this study, instead of using different concentrations, the variable was the contact time of the nanofibers with the embryo development medium and one and 10-h trials were carried out. Therefore, the determined incubation time can be used as the IC50 dose similar to the drug toxicity studies and thus the safe contact time with the sample can be determined. Falinski et al. stated that while determining the toxicity of nano-sized materials, the accumulation and effect of nano-sized materials in biological systems should be evaluated under appropriate system conditions to increase the usefulness and accuracy of bioanalysis in aqueous media (Falinski et al., 2019). Accordingly, in this study, MDA, NO levels and GST, SOD activities were determined in zebrafish embryo homogenates to investigate whether lactase enzyme or anti-BPA antibody immobilized PCL/SF-based nanofibers cause oxidative damage in zebrafish embryos. MDA and NO levels were tested to detect the extent of oxidative damage. Raw nanofiber did not increase oxidant damage, but enzyme or antibody-immobilized nanofibers slightly increased MDA levels. MDA is a byproduct of lipid peroxidation, in which reactive oxygen species (ROS) disrupt the structure of lipids in cell membranes. An increase in MDA levels in the presence of bioactivated nanofibers suggests that zebrafish embryos may be more susceptible to oxidative stress and cellular damage as a result of the lactase enzyme, anti-BPA antibody, or immobilization technique and its chemicals. These findings demonstrate that zebrafish embryos exposed to bioactivated nanofibers

suffer oxidative stress. NO is a signaling molecule and has a role in immunological responses and vasodilation. Reduced NO levels following nanofiber exposure may be a symptom of an impaired cellular signaling system or a decreased capacity to defend against oxidative stress, maybe as a result of the oxidative damage carried on by the nanofibers. SOD is an important antioxidant enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. A reduction in SOD activity in the bioactivated nanofiber exposure groups may indicate that the SOD enzyme is being consumed by excessive reactive oxygen species or that its expression is being down-regulated as a result of cellular stress responses. GST is another important enzyme in the detoxification process, converting reduced glutathione into xenobiotic substrates for detoxification. Lower GST levels following nanofiber exposure indicate an impaired detoxification mechanism, which might be related to cellular glutathione depletion or enzyme inhibition.

Although a minor oxidant-antioxidant imbalance was identified, the enzyme or antibody-immobilized nanofibers had no damaging effects when kept in an embryo development medium for 1 h, and they caused no deformation or mortality in zebrafish embryos. The findings of this study are consistent with previous research on the oxidative effect of nanomaterials, emphasizing the need for testing such effects for the safe use of bioactive nanofibers in food applications. Studies on zebrafish embryos exposed to different nanomaterials revealed that oxidative stress increased as antioxidant enzyme activity decreased (Lu et al., 2022; Mahjoubian et al., 2023).

The findings of this study show that lactase-immobilized PCL/SF-based nanofibers and anti-BPA antibody nanofibers are biocompatible with zebrafish embryos under the conditions tested, despite the presence of oxidative stress indicators that indicate a response to the nanofibers.

In conclusion, the biocompatibility of these two bioactive nanofiber-based biomaterials that would come into contact with food was tested by integrating zebrafish embryos as an *in vivo* biocompatibility test model. Therefore, the methods used in this study can contribute to the reliability of newly developed biomaterials in terms of *in vivo* biocompatibility tests optimized for human use.

Consent to participate

All the authors have agreed for authorship, read and approved the manuscript, and given consent to participate.

Ethics approval

As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC.

CRediT authorship contribution statement

Güzin Göksun Sivas: Methodology, Investigation. **İsmail Ünal:** Methodology, Investigation. **Begüm Gürel-Gökmen:** Methodology, Investigation. **Ebru Emekli-Alturfan:** Validation, Resources, Methodology, Conceptualization. **Tuğba Tunalı Akbay:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors report no conflicts of interest.

Data availability

Data will be made available on request.

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