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Research Article

ANTIOXIDANT POTENTIAL OF BILIRUBIN NANOPARTICLES: *IN VITRO* TIME AND CONCENTRATION DEPENDENT STUDY

Dhaval J Kamothi, Vinay Kant*, Santosh D Dev Yadav, Vinod Kumar

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ABSTRACT: Many diseases are caused by oxidative stress. Protein, lipid, and DNA macromolecules are harmed by extremely unstable radicals (free radicals) like superoxide anion and hydrogen peroxide, and reactive nitrogen species. Oxidative stress also aggravates inflammation in response to inflammatory agonists by activating NF-kB. The antioxidant activity of bilirubin nanoparticles was investigated in this work. Bilirubin was entrapped in pluronic F-127 (PF-127) and bilirubin nanoparticles (BNP) having size 100-150 nm were synthesized. TEM studies revealed that the BNP has spherical morphology. The antioxidant potential of BNP was evaluated by DPPH as well as ABTS assay at different incubation periods. BNP showed higher radical scavenging activity in comparison to bulk bilirubin (bulk B) in both DPPH as well as ABTS assays. Inhibitory concentration50 (IC50) of 0.1% BNP was the lowest in comparison to other formulations at all the incubation periods in the DPPH assay. The IC50 of 0.03% BNP was the lowest in comparison to other formulations at all incubation periods in the ABTS assay. Additionally, BNP (0.03, 0.1, and 0.3%) showed a decrease in the IC50 with an increase in the incubation period in both DPPH as well as ABTS assay. These observations support the potential of bilirubin nanoparticles as a novel antioxidant compound in the future.

Keywords: Bilirubin nanoparticles, Pluronic F-127, DPPH, ABTS, Inhibitory concentration50, Scavenging activity.

INTRODUCTION

The evaluation of nano-materials for antioxidant activity has become an important and basic study in nanoscience and technology. Molecules with greater reactivity than molecular oxygen are known as reactive oxygen species (ROS) [1, 2]. These molecules are made up of extremely reactive radicals like superoxide anion [3] and hydrogen peroxide [4, 5]. ROS serves a role in maintaining homeostasis and primarily operates in modifying cellular dysfunction, which contributes to disease pathogenesis [6, 7]. Numerous enzymatic reactions occurring in various cellular constituents, such as the cytoplasm, endoplasmic reticulum, peroxisome, and mitochondria, result in the generation of ROS as secondary products. NOXes referred to as NADPH oxidases are enzymes that particularly create ROS. Diabetes is associated with oxidative stress, which includes increased free radical production and decreased antioxidant activity, resulting in vascular abnormalities [8]. An array of chronic illnesses in humans including cancer, myocardial infarction, rheumatoid arthritis, cardiovascular diseases, atherosclerosis, chronic inflammation, stroke, post-ischemic perfusion injury and septic shock, and other degenerative diseases, are brought on by unchecked production of ROS, which oxidatively damages lipids, proteins, and DNA [9, 10]. ROS triggers NF-κB in the presence of inflammatory agonists [11]. This, in turn, triggers the induction of inflammatory genes, inflammasome activation, and cytokine secretion. Nevertheless, the body contains antioxidants, and those acquired from food counteract the impact of ROS. Antioxidants are defined as extrinsic (natural or synthetic) or intrinsic substances that are involved in the neutralization of radicals, functioning as scavengers of ROS or their precursors [12]. The body has a

Department of Veterinary Pharmacology and Toxicology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India.
**Corresponding author. e-mail: drvinaykantluvas@gmail.com*

system that protects against oxidant-mediated cellular damage, but metabolic illnesses cause this mechanism to malfunction, which increases free radical production and causes cellular damage.Therefore, there is a need for a compound that has excellent antioxidant as well as anti-inflammatory activity.

Bilirubin containing tetrapyrrole ring exists in different forms in blood: as direct bilirubin when it's conjugated with glucuronic acid, as indirect bilirubin when it's bound to serum albumin, and as free bilirubin when it's unconjugated and unbound [13]. Studies both *in vivo* and *in vitro* have shown that bilirubin neutralizes ROS at low concentrations, which lessens oxidative stress [14, 15, 16]. It reduces ROS and has been proven to stimulate wound healing in diabetic patients [17]. Higher serum bilirubin decreases the risk of diabetes mellitus nephropathy [18, 19]. Bilirubin mitigates the pancreatic damage induced by streptozotocin in Gunn rats [20] and protects rodents against diabetic nephropathy by suppressing oxidative stress and reducing the expression of NADPH oxidase [21]. Although bilirubin is an effective antioxidant and cytoprotectant, its full potential is not exploited due to its hydrophobic nature, insolubility, large particle size, and shorter half-life, hence its clinical application is limited [22, 23, 24]. In our earlier work, the wound healing effect [25] as well as the antibacterial activity [26] of BNP were investigated. Given this, nanotechnology could offer a different way to get over the obstacles and fully utilize bilirubin's antioxidant and cytoprotective properties. Thus, in the current investigation, novel bilirubin nanoparticles have been synthesized to assess their antioxidant activity relative to their bulk form.

MATERIALS AND METHODS

Materials

Bilirubin, PF-127, and DMSO were acquired from Sigma-Aldrich. Eudragit gifted by Evonik Industries. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were acquired from SRL India.

Synthesis of BNP

The nanoparticles were synthesized following the standard method with some modifications [27]. Bilirubin and eudragit were dissolved in DMSO and stirred for 20 minutes. Following this, PF-127 (1%) aqueous solution was formulated. Subsequently, the bilirubin solution was slowly added to PF-127. The mixture was stirred magnetically at 1000 rpm for 2 hours to facilitate encapsulation. Finally, the suspension was subjected to 5 minutes of ultrasonication at 4°C. BNP of three concentrations including (0.03%, 0.1%, and 0.3%) were prepared. The bilirubin-to-eudragit ratio was maintained at 1.0 : 0.5.

Characterization techniques

Hydrodynamic diameter along with polydispersity index (PDI)

Zetasizer (Malvern, UK) determined the parameters following the instrument's instructions.

Zeta potential estimation

Zetasizer (Malvern, UK; Ver. 7.11, Model no.: MAL1085352) was used to measure the nanoparticles' zeta potential following the instrument's instructions. To ascertain the surface charge of the BNP, zeta potential measurements were made. The zeta cell was introduced with the BNP using a syringe. The zeta cell was put in the device after the zeta cell was filled with the nanosuspension [28].

TEM study

Using TEM, the morphology and size of nanoparticles were ascertained. For the investigation, a grid was utilized, upon which the suspension of dispersed nanoparticles was deposited before being moved to the microscope.

Antioxidant potentials of BNP

Assay for DPPH

BNP's ability to scavenge DPPH was assessed using the standard method with some modifications [29, 30]. In this assay, a stock solution of DPPH (0.2 mM) (Cat. no. 29128, SRL, India, MW 394.32 g/mol) in methanol was used. DPPH (4 mg) was dissolved in methanol (50ml). For this experiment, a 96-well plate was used. Each row's well number one received DPPH solution (280 µl) and 200 µl DPPH solution in other wells of the plate. In well no. 1 of each row of the plate, 20 µl of one of the different preparations [*i.e.* PF-127, blank NP, bulk B, BNP] or antioxidant *i.e.* 0.3% ascorbic acid (AA) and 0.3% butylated hydroxytoluene (BHT) was added leading to a total volume of 300 µl. Thereafter, three times serial dilutions (from 1:3 to 1:59049) of different preparations and standard antioxidants were prepared in the DPPH solutions. For this, 100 µl from well no. 1 (having 280 µl DPPH and 20 µl preparation/standard) of each row was transferred to well no. 2 (containing 200 µl of DPPH) followed by 100 µl from well no. 2 to well

no. 3. Similar further serial dilutions were done in the wells upto well no. 11 of plate. The 100 µl from well no. 11 was discarded, which led to an equal volume of 200 µl in each of the wells. The well no. 12 of each row contains only 200 µl DPPH solution (without any sample preparation or antioxidant), which served as a control. After 30 minutes, the readings were taken at a wavelength of 517 nm using an ELISA reader. The plate was further kept at dark and absorbance was again measured at 45, 75, 120, and 150 min at 517 nm. This was done to determine the change in the antioxidant effect of different bilirubin nanoformulations concerning time due to the release of bilirubin from the nanoparticles. The protocol was done three times. DPPH scavenging percent of the preparations and standards were calculated as follows:

% DPPH scavenging = [Absorbance (DPPH) - Absorbance (test)] / [Absorbance (DPPH)] X 100

Values of DPPH scavenging activities of the optimum dilutions (0.011 to 74.00 µg/ml) of BNP along with standards were used to calculate the inhibitory concentration50 (IC50) values (*i.e.* drug concentration at which 50% inhibition of radicals occurred) against DPPH radicals at 30, 45, 75, 120, and 150 min with the aid of GraphPad (v8.0.2).

Assay for ABTS

BNP's ability to scavenge ABTS was assessed using the standard method with some modifications [30, 31]. In this assay, a stock solution of ABTS (2 mM) (Cat. no. 28042, SRL, India, MW 548.68 g/mol) and potassium persulfate (70 mM) were used. To prepare ABTS (2 mM) stock solution, 109 mg was added to 100 ml distilled water. Potassium persulfate (70 mM) was prepared by adding 1.892 g to 100 ml of distilled water. ABTS radicals were generated by mixing ABTS solution with potassium persulfate. The mixture was stored for 6 to 12 hours. ABTS is then diluted with PBS (1:5). Within the 96-well plate, ABTS (280 µl) diluted solution was included within well no. 1 of each row, and 200 µl in other wells of the plate. In well no. 1 of each row of the plate, 20 µl of one of the different preparations [*i.e.* PF-127, blank NP, bulk B, BNP] or antioxidant *i.e.* 0.3% ascorbic acid (AA) and 0.3% butylated hydroxytoluene (BHT) was added leading to a total volume of 300 µl. Thereafter, three times serial dilutions (from 1:3 to 1:59049) of different preparations and standard antioxidants were prepared in the ABTS solutions. For this, 100 µl from well no. 1 (having 280 µl ABTS and 20 µl preparation/standard) of each row was transferred to well no. 2 (containing 200 µl of ABTS) followed by 100 µl from well no. 2 to well no. 3. Similar further serial dilutions were done in the wells upto well no. 11 of plate. After 5 minutes, the readings were taken at a wavelength of 734 nm using an ELISA reader. The plate was further kept at dark and absorbance was again measured at 20, 50, 90, and 120 min at 734 nm. The 100 µl from well no. 11 was discarded, which led to an equal volume of 200 µl in each of the wells. Well no. 12 of each row contains only 200 µl ABTS diluted solution (without any sample preparation or antioxidant), which served as control. This was done to determine the change in the antioxidant effect of different bilirubin nanoformulations concerning time due to the release of bilirubin from the nanoparticles. The protocol was done three times. ABTS scavenging percent of the preparations and standards were calculated as follows:

% ABTS scavenging = [Absorbance (ABTS) - Absorbance (test)] / Absorbance (ABTS) X 100

Values of ABTS scavenging activities of the optimum dilutions (0.011 to 74.00 µg/ml) of BNP along with standards were used to calculate the inhibitory concentration50 (IC50) values (*i.e.* drug concentration at which 50% inhibition of radicals occurred) against ABTS radicals at 5, 20, 50, 90, and 120 min with the aid of GraphPad (v8.0.2).

Statistical Analysis

With n being the number of replicates, the values are shown as mean ± SEM. A two-way ANOVA was used to compare and examine the values of the different groups. Following that, GraphPad Prism was used to conduct a suitable post-test (Dunnett's). At p less than 0.05, the differences between the treatment groups were deemed statistically significant.

RESULTS AND DISCUSSION

A new category of materials called nanomaterials is in high demand for a variety of practical uses. They are solid, colloidal particles having hydrodynamic diameters ranging from 1-1000 nm, although, for biomedical applications, nanoparticles having sizes less than 200nm are usually synthesized [32]. Studies are performed to use different nano-particles in healthcare, singly or in different combinations [33, 34, 35]. In the current study, bilirubin nanoparticles having

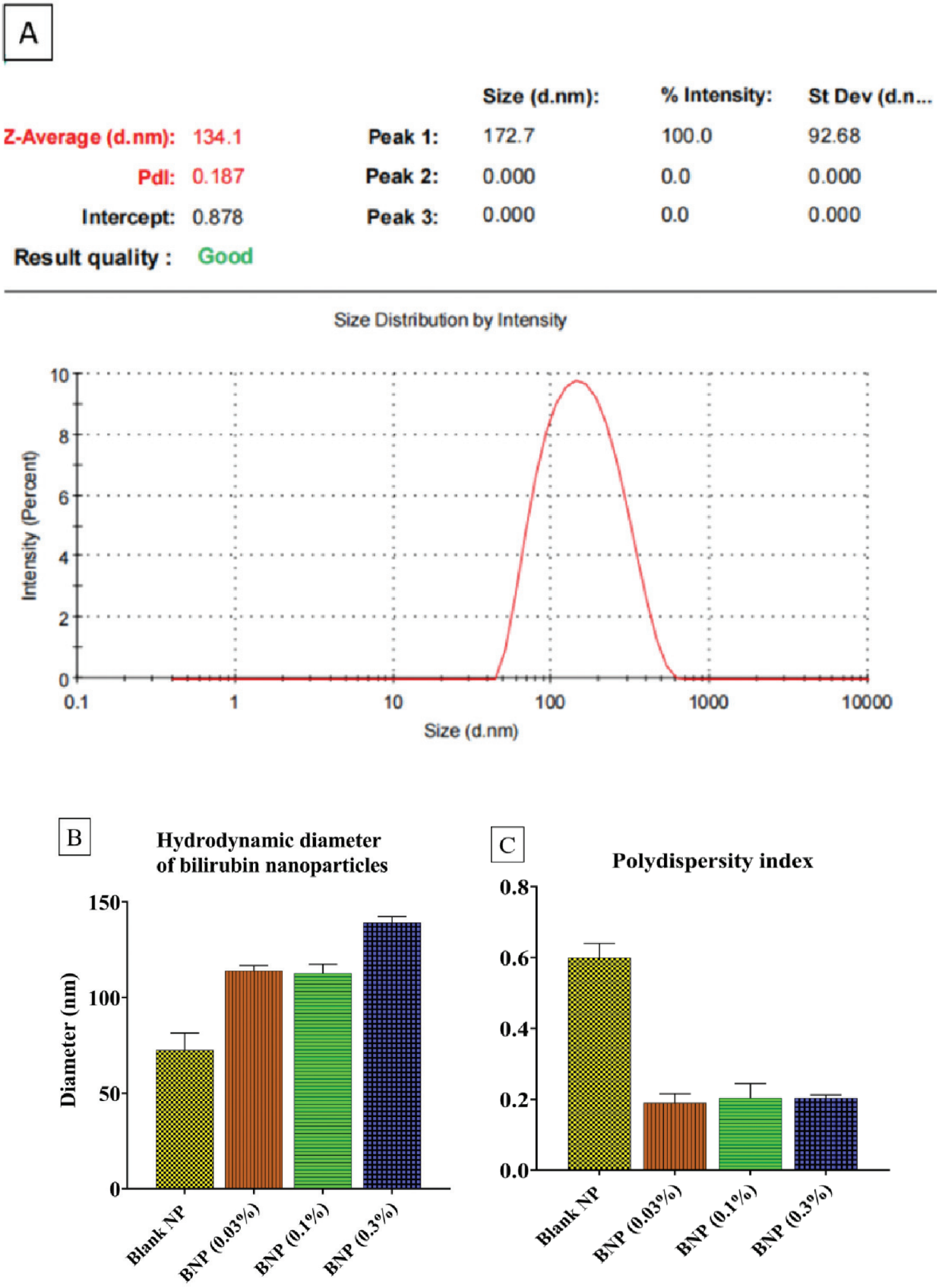

Fig. 1. (A) Hydrodynamic diameter and PDI of BNP generated by Zetasizer. (B) Hydrodynamic diameter of blank NP and BNP (C) PDI of blank NP and BNP. B & C: Data are presented as mean ± SEM (n=3).

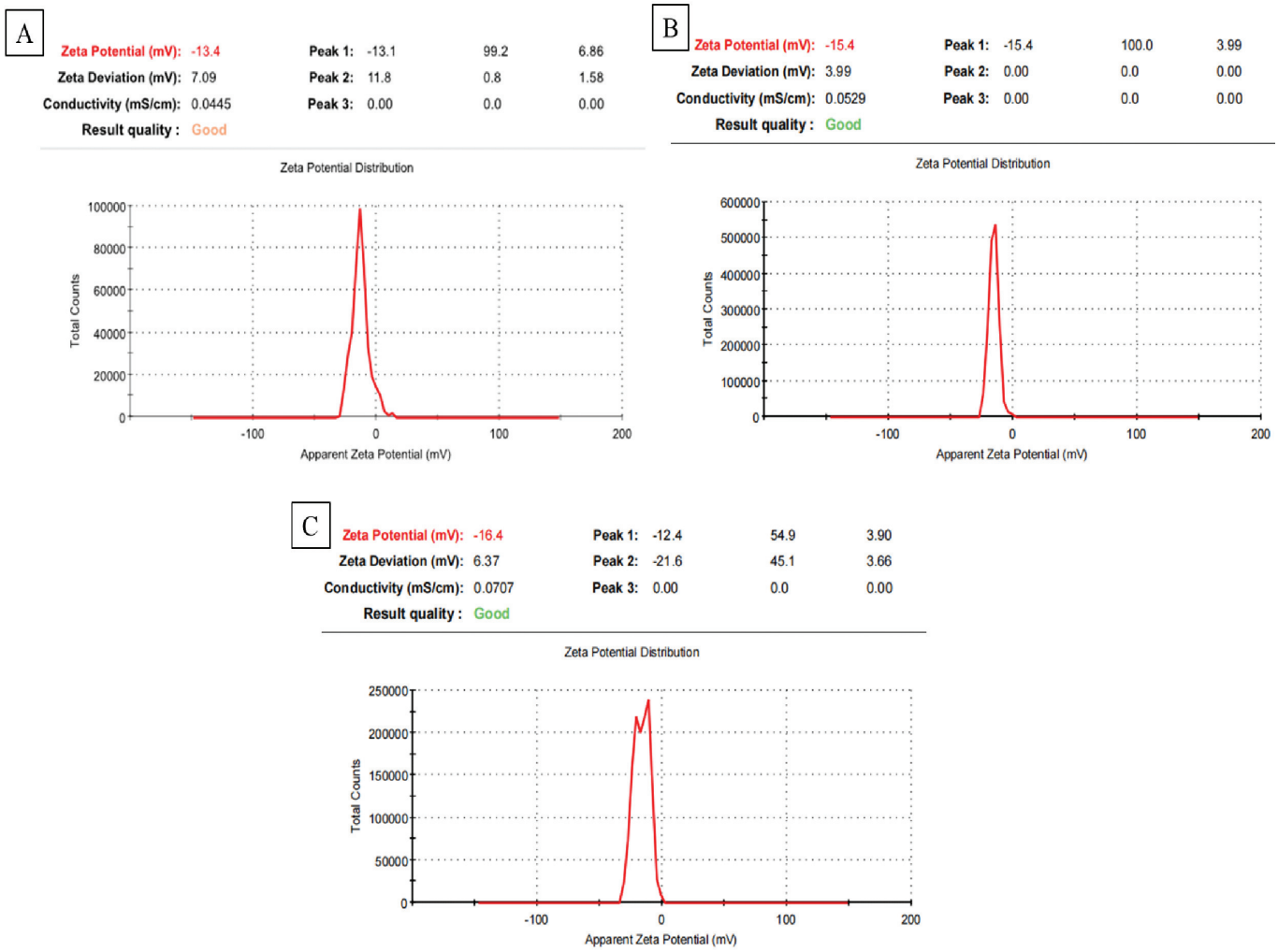

Fig. 2. Zeta potential of BNP generated by zetasizer. (A) 0.03% BNP (B) 0.1% BNP and (C) 0.3% BNP.

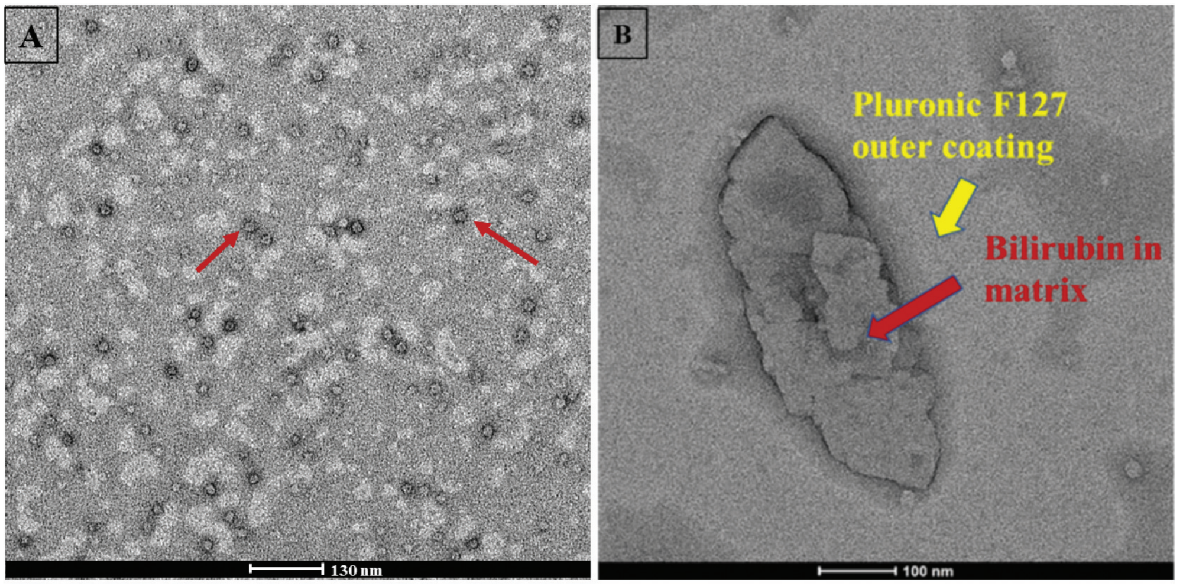

Fig. 3. (A) TEM of BNP (lower magnification) (B) BNP showing the outer cover of PF-127 and bilirubin core (higher magnification).

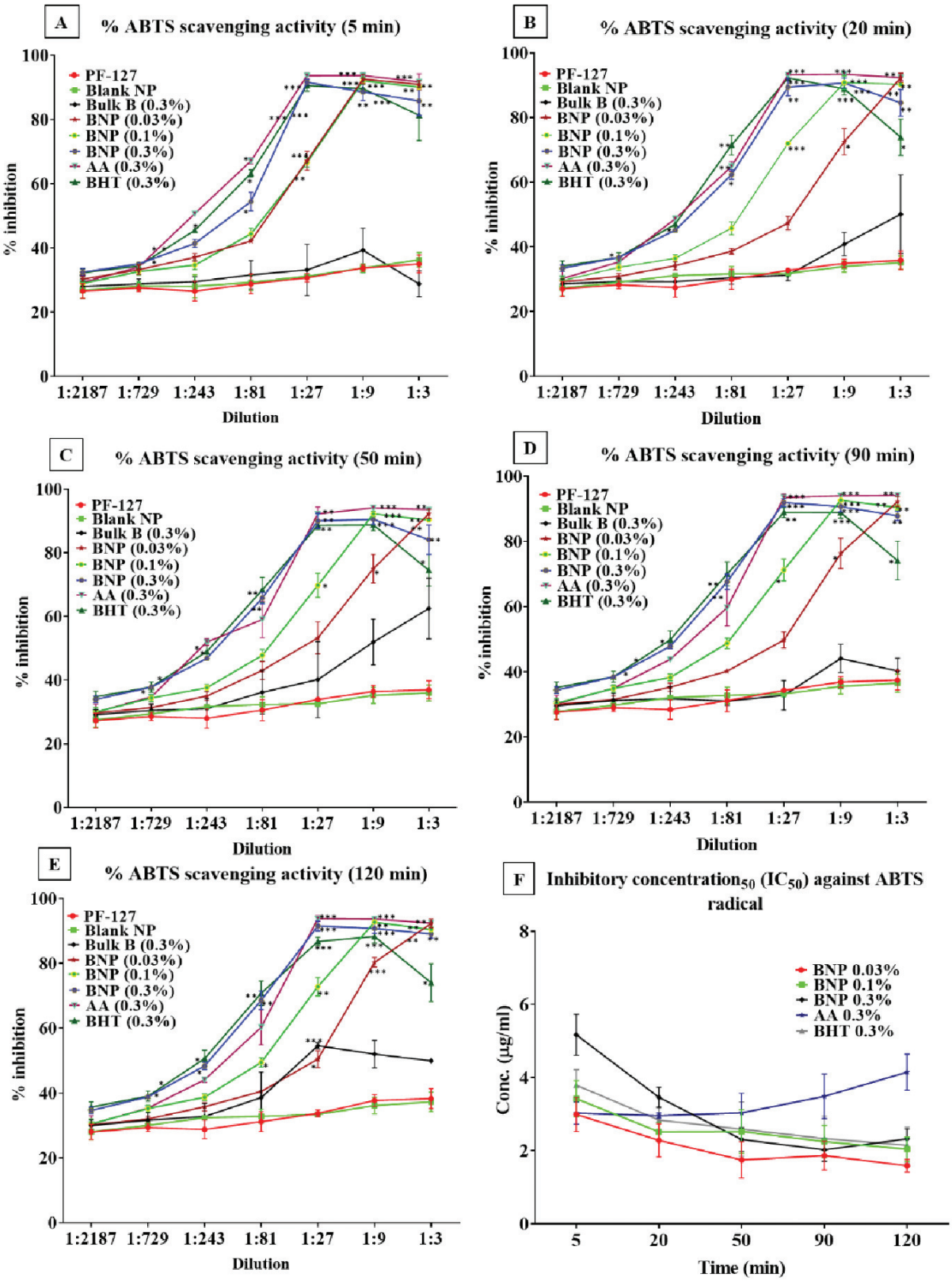

Fig. 4. (A-E) Percent DPPH scavenging activity of different formulations at different dilutions after incubation for different time intervals and (F) IC50 values at different incubation period. Data are presented as mean ± SEM (n=3) and *, **, *** denote p less than 0.05, 0.01 and 0.001, respectively vs PF-127 in Fig. A-E. In Fig. F, data are presented as mean ± SEM (n=3) and * represents p less than 0.05 vs BHT].

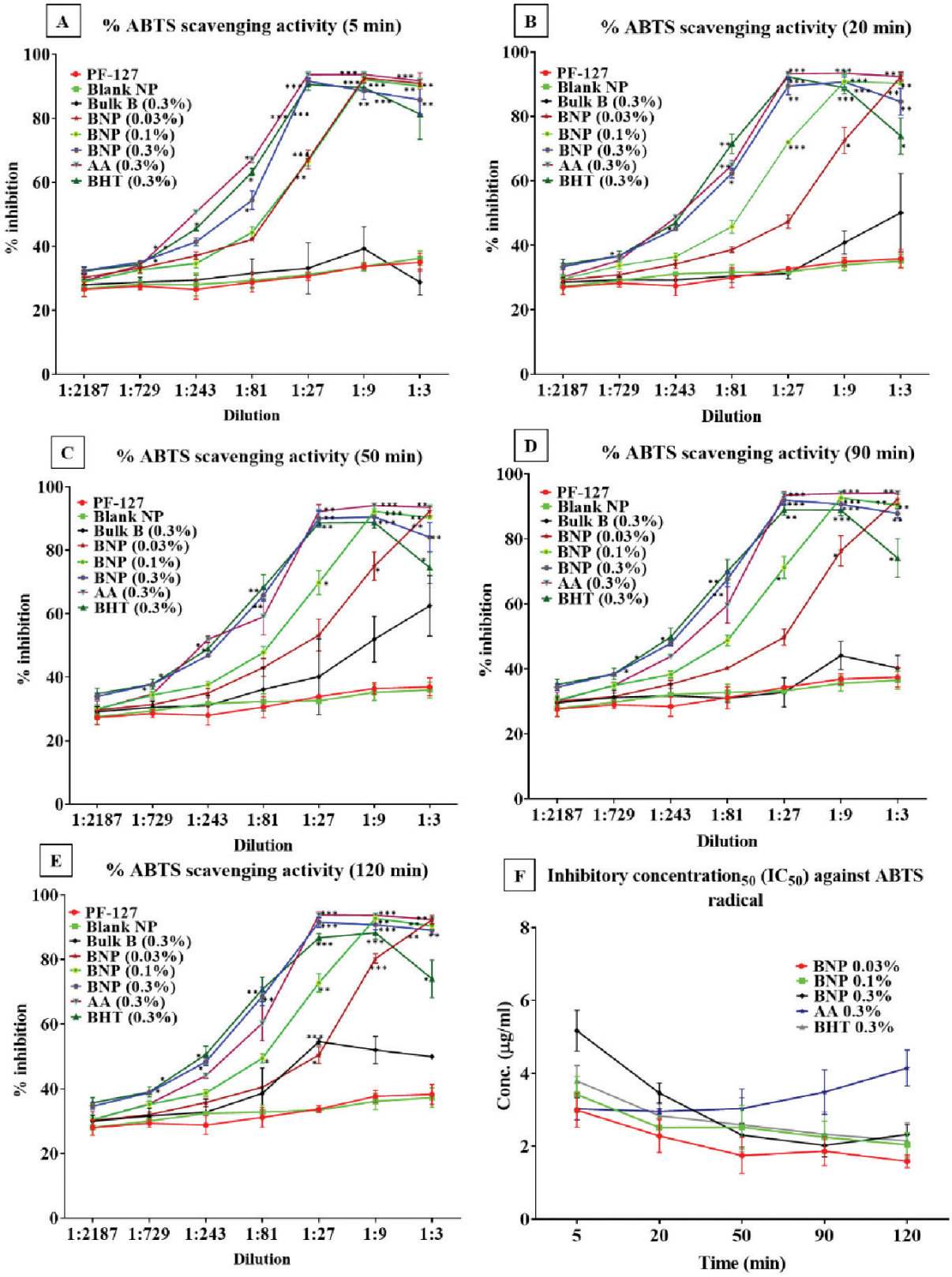

Fig. 5. (A-E) Percent ABTS scavenging activity of different formulations at different dilutions after incubation for different time intervals and (F) shows IC50 values at different incubation period. Data are presented as mean ± SEM (n=3), and *, **, *** represent p less than 0.05, 0.01 and 0.001, respectively vs PF-127.

hydrodynamic diameters ranging from 100 to 140 nm were synthesized. Fig. 1A is the representative graph showing the size along with the PDI of BNP generated by Zetasizer. Average size & PDI of blank NP and BNP (0.03%, 0.1% and 0.3%) are presented in Fig 1B & Fig 1C, respectively. The particle size of the blank NP was 72.57±8.87 nm and that of BNP at 0.03%, 0.1%, and 0.3% concentrations was found to be 113.93±2.73 nm, 112.70±4.68 nm, and 139.23±3.08 nm, respectively. PDI of blank NP was found to be 0.6 ± 0.04 and that of BNP at 0.03%, 0.1%, and 0.3% concentrations was found to be 0.19 ± 0.03, 0.20 ± 0.04 & 0.20 ± 0.01, respectively.

Nanoparticles have controlled release property, which prevents active compounds from degrading and preserves their function while also increasing biological availability [36, 37]. A tri-block copolymer called pluronic F127 that is categorized as polymorph material contains polyethylene oxide (PEO) and polypropylene oxide (PPO) arranged as [PEO]–[PPO]–[PEO] structure. The central PPO block turns hydrophobic, while the PEO blocks retain their hydrophilic nature at elevated temperatures [38]. The aggregation of amphiphilic moieties leads to the spontaneous production of polymeric micelles, with the hydrophobic moieties forming the micellar core and the hydrophilic ends forming the structural shell. Pluronics having such an adaptive structure have been recognized as an important drug carrier due to their applications as emulsifiers, solubilizing agents, and dispersive ingredients for pharmaceutical formulation. Bilirubin nanoparticles with hydrodynamic diameters of 100-150 nm were synthesized in the current study by encapsulating bilirubin in pluronic F-127, as validated by zeta sizer and TEM analysis. The zeta potential of bilirubin nanoparticles ranged from -13 to -18 mV, demonstrating a stable nanoformulation and agreeing with the published value [27]. BNP's zeta potential was -13.81±0.18mV, -15.49±0.13mV & -16.83±0.11mV at 0.03%, 0.1%, and 0.3% concentrations, respectively (Fig. 2).

The morphology of the BNP was revealed to be spherical in the TEM study (Fig. 3). BNP was of nano-size having a spherical shape which was also observed in zeta sizer analysis. The images showed a cloudy outer covering of pluronic F127 encapsulating bilirubin in its matrix.

Bilirubin possesses antioxidant and anti-inflammatory characteristics, and it has an inverse association with obesity, hypertension, diabetes, and cardiovascular diseases [39]. Earlier, bilirubin has shown potent antioxidant activities in a concentration (5-25 μM) dependent manner to scavenge DPPH radicals [40]. In earlier studies, bilirubin or its nanoforms have shown their *in vivo* antioxidant effect [41, 42]. PEGylated bilirubin nanoparticles have protected pancreatic islets against paraquat-induced ROS [43]. As a result, in the current work, an *in vitro* investigation was performed to analyze the antioxidant effect of bilirubin nanoparticles at various incubation times. The DPPH scavenging effect of various formulations, including PF-127, bulk B, blank NP, BNP, ascorbic acid, and BHT, was concentration-dependent at different times of incubation (Fig. 4A-E). Most of the formulations showed optimum inhibition at 1 : 3 to 1 : 2187 dilutions. The formulations did not show marked scavenging activity beyond 1 : 2187 dilutions. The 0.3% bilirubin nanoparticles showed decreased activity at 1:3 dilutions concerning 1 : 9 dilutions. At 30, 45, 75, 120, and 150 min of incubation, the 1:9 to 1:81 dilution of bilirubin nanoparticles (0.03, 0.1 and 0.3%), ascorbic acid (0.3%) and BHT (0.3%) showed significantly higher percent scavenging activity against DPPH in comparison to bulk bilirubin. Bilirubin nanoparticles also showed an increase in the DPPH scavenging with an increase in the incubation period. PF-127, bulk B and blank NP failed to produce even 50% scavenging activity at different dilutions after various incubation periods. Ascorbic acid showed the maximum scavenging activity against DPPH at all different incubation periods in comparison to other formulations. The IC50 of 0.1% bilirubin nanoparticles was the lowest in comparison to other formulations at all the incubation periods. Additionally, the bilirubin nanoparticles (0.03, 0.1, and 0.3%) showed a decrease in the IC50 values with an increase in the incubation period *i.e.* from 30 min to 150 min (Fig. 4F).

The ABTS scavenging impact of numerous formulations, including PF-127, bulk B, blank NP, BNP, ascorbic acid, and BHT, was concentration-dependent at different times of incubation (Fig. 5A-E). Most of the formulations showed optimum ABTS scavenging activity at 1:27 to 1:2187 dilutions. The formulations did not show marked ABTS scavenging activity beyond 1:2187 dilutions. Bilirubin nanoparticles (0.03, 0.1, and 0.3%), ascorbic acid (0.3%), and BHT (0.3%) showed significantly or markedly higher percentage of ABTS scavenging activity in comparison to pluronic F-127 after incubation for different periods. Bilirubin nanoparticles also showed an increase in the ABTS scavenging

activity with an increase in the incubation period. PF-127, bulk B and blank NP failed to show 50% scavenging activity. To compare the IC50 of different formulations, different ranges of dilutions were selected representing the same concentration *i.e.* (0.011 µg/ml to 74.00 µg/ml) for each formulation having a distinct percentage inhibition of radicals at that particular range of concentration. Following this, percentage inhibition data was plotted against the particular concentration in GraphPad Prism v8.0.2 software program to estimate IC50 for each formulation. The procedure was followed for all the incubation periods. The ABTS assay revealed that IC50 of 0.03% bilirubin nanoparticles was the lowest in comparison to other formulations at all incubation periods. Additionally, the bilirubin nanoparticles (0.03, 0.1, and 0.3%) showed a decrease in the IC50 values with an increase in the incubation period *i.e.* from 5 min to 120 min (Fig. 5F).

In comparison to bulk bilirubin, BNP inhibited DPPH & ABTS radicals significantly more. The 0.3% bilirubin nanoparticles showed lower scavenging activity in comparison to that of 0.03% and 0.1% bilirubin nanoparticles at 1:3 dilution. This might be due to the decreased release of bilirubin from nanoparticles at higher concentrations, which reduces the ability of bilirubin to trap DPPH & ABTS radicals. This was further revealed by the increased scavenging of DPPH & ABTS radicals by 0.3% bilirubin nanoparticles at 1:9 dilution in comparison to 1:3 dilution. IC50 of bilirubin nanoparticles against DPPH & ABTS radicals mostly decreased concerning the increase in the incubation period. This indicates sustained release and time-dependent scavenging effect of bilirubin nanoparticles against DPPH & ABTS radicals. Bilirubin can act as a reducing agent and neutralize oxygen radicals [44]. This demonstrated that bilirubin's capacity to donate electrons may be the cause of its DPPH & ABTS scavenging action. It was found that the bilirubin nanoparticles showed significant antioxidant activity in comparison to the bulk bilirubin against DPPH & ABTS radicals. This indicates that the bilirubin being a potent molecule did not exhibit significant antioxidant activity in its bulk form. This could be due to low solubility and aggregation of bulk bilirubin which interfered with the interaction between the radicals and bilirubin, resulting in its lower antioxidant effect as compared to the bilirubin nanoparticles. Earlier the concentration-dependent antioxidant effect of bilirubin was evaluated, therefore, in the current study, a newer approach was adopted by synthesizing bilirubin nanoparticles and evaluating its scavenging activity against DPPH & ABTS taking into account the concentration and time-dependent effect.

CONCLUSION

Considering the outcomes of this work, it is possible to conclude that PF-127 could be an appropriate nanocarrier. Synthesized novel BNP showed significantly better antioxidant activity in both DPPH & ABTS assay as compared to bulk B. BNP also showed an increase in the radical scavenging effect with an increase in the incubation period. The IC50 of BNP was the lowest as compared to other formulations at all incubation periods. Additionally, the BNP showed a reduction in the IC50 with an increase in the incubation period, which indicates a controlled release of the bilirubin from PF-127 nanoparticles. Thus, BNP could be envisioned as a new antioxidant formulation in metabolic and cardiovascular illnesses.

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