PREPARATION AND CYTOTOXICITY OF NOVEL BIOACTIVE COMPOUND-CONTAINING POLYMERIC NANOSIZED MATERIALS

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ABSTRACT

Novel nonwoven textile from polylactide and Schiff base from Jeffamine ED® and 8-hydroxyquinoline-2-carboxaldehyde (Jeff-8Q) or its complex with Cu^{2+} (Jeff-8Q.Cu^{2+}) of diverse design was fabricated by one-pot electrospinning or electrospinning combined with dip-coating. Stable aqueous dispersions of NPs based on complexes of Brb and poly(methacrylic acid) or poly(acrylic acid), were also successfully prepared by mixing their dilute aqueous solutions. The morphology of the nanosized materials (fibrous mats and NPs) were studied by scanning electron microscopy (SEM) or transmission electron microscopy (TEM). In vitro cell viability studies and used fluorescent staining methods demonstrated that the prepared Jeff-8Q- or Jeff-8Q.Cu^{2+}-containing fibrous materials and Brb-containing NPs displayed higher cytotoxicity against HeLa tumor cells than against non-tumor BALB/c 3T3 mouse fibroblast cells. These properties render these novel polymeric nanosized materials promising as potential candidates in the drug delivery systems in the treatment of cervical tumors.

1. INTRODUCTION

It is well known that 8-hydroxyquinoline, its derivatives and natural isoquinoline quaternary alkaloid berberine chloride (Brb) manifest a set of beneficial biological properties: antimicrobial, antioxidant, antitumor, etc. [1-4]. However, Brb has some drawbacks. It is poorly soluble in water and body fluids and its intestinal absorption is very low, which predetermine its low bioavailability. Incorporation of Brb in polymer matrix is expected to contribute to obviating these drawbacks.

In the recent years, polymeric nanosized materials (electrospun nonwoven textile and nanoparticles (NPs)) have evoked considerable interest as carriers of bioactive compounds with the potential to overcome issues such as poor water solubility of these compounds as well as their low bioavailability. The specific properties of these materials related to their nanoscale size and the possibility for sustained release of the bioactive compounds lead to a decrease in the cytotoxicity and an improvement of the therapeutic effect of the compounds. The use of NPs from natural and synthetic polymers for encapsulation of Brb and for enhancing its bioavailability has been reported [5-9]. It has been shown that electrospun fibrous materials are suitable carriers for 8-hydroxyquinoline derivatives. These derivatives have been encapsulated into electrospun nonwoven textile from polyvinyl alcohol [10], polylactide/poly(ethylene glycol) blend or poly(ethylene glycol) grafted on polylactide [11], polylactide/poly(butylene succinate) [12], chitosan/poly(ethylene oxide) [13], *N*-carboxyethyl chitosan/polyacrylamide [14], cellulose acetate or cellulose acetate/ poly (ethylene glycol) [15], etc.

The aim of the present study was to prepare novel electrospun nonwoven textile containing 8-hydroxyquinoline derivative and its complex with Cu^{2+} and NPs containing Brb. The effect of the

composition of the obtained materials on their cytotoxicity against HeLa tumor cells and non-tumor BALB/c 3T3 mouse fibroblast cells was estimated.

2. Experimental

2.1. Materials

Polyetheramine (Jeffamine ED®-2000, further denoted as Jeff) (Aldrich (St. Louis, MO, USA)), 8-hydroxyquinoline-2-carboxaldehyde (8Q) (Aldrich (St. Louis, MO, USA)), $CuCl_2$ (Aldrich (St. Louis, MO, USA)), berberine chloride (98%) (Brb) (Sigma-Aldrich, India) and poly(acrylic acid) (PAA) (M_w 250000 g/mol) (Aldrich, Gillingham, Dorset-England) were with analytical grade of purity and were used without further purification. PMA was prepared by radical polymerization as previously described [16]. The average viscometric molar mass (M_v) of PMA (7.4 $\times 10^5$) was determined in 0.002 M HCl at 30 °C using the equation: $[\eta] = 6.6\ 10{-}4\ M_v^{0.5}$ [17]. Poly(L-lactide-co-D,L-lactide) (Polylactide, PLA) Resomer® LR 708 (M_w 911 000 g/mol, M_w/M_n=2.46) - (L-lactide:D,L-lactide molar ratio = 69:31) was kindly donated by Boerhinger-Ingelheim Chemicals Inc. (Ingelheim am Rhein, Germany). The culturing of HeLa tumor cells was performed as previously described [18]. A cell culture of non-tumor BALB/c 3T3 mouse fibroblast cells was cultivated as previously described [19]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide (EtBr), and acridine orange (AO) were purchased from Sigma-Aldrich, Schnelldorf, Germany. All culture reagents Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Schnelldorf, Germany), fetal bovine serum (FBS) (Gibso/BRL,Grand Island, NY), glutamine, penicillin and streptomycin (LONZA, Cologne, Germany) were used as received.

2.2. Preparation of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile

2.2.1. Preparation of Jeff-8Q/inPLA and Jeff-8Q.Cu^{2+}/inPLA mats by one-pot electrospinning (type "in" nonwoven textile). The 8-hydroxyquinoline derivative used in the present study was Schiff base from Jeffamine ED® and 8-hydroxyquinoline-2-carboxaldehyde (further denoted as Jeff-8Q). Jeff-8Q was prepared as described earlier [20]. DMF/DMSO mixture (60/40 v/v) was used as solvent for the spinning solutions. Jeff-8Q-loaded PLA nonwoven textile (with a PLA/Jeff-8Q weight ratio of 70/30) was prepared by electrospinning of mixed Jeff-8Q/PLA solutions at polymer concentration 5 wt%. The PLA fibrous materials with Jeff-8Q loaded in the bulk will be further denoted as Jeff-8Q/inPLA. The complex of Jeff-8Q with Cu^{2+} was prepared by mixing the 1.6 ml 8 wt% solution of Jeff-8Q with 1 ml 1.4 wt% solution of $CuCl_2$ at a molar ratio of 8Q end groups of Jeff-8Q and Cu^{2+} of 1:1. PLA mats containing complex of Jeff-8Q with Cu^{2+} in the bulk will be further denoted as Jeff-8Q.Cu^{2+}/inPLA. Jeff-8Q.Cu^{2+}/inPLA fibers with a weight ratio of PLA/Jeff-8Q.Cu^{2+} = 70/30 were obtained by electrospinning of their mixed solutions at polymer concentration of 5 wt.%.

The electrospinning was performed using a set-up consisting of a high voltage power supply; a pump [NE- 300 Just Infusion™ Syringe Pump (New Era Pump Systems Inc., USA)] for delivering the spinning solution at a constant rate; a syringe provided with a positively charged metal needle and a custom-made grounded rotating drum collector. Electrospinning was carried out under the following conditions: flow rate of 1.0 ml/h, voltage of 25 kV, needle tip-to-collector distance 20 cm, collector rotating speed of 1500 rpm, ca. 49% relative humidity, and room temperature (25 °C). The electrospun fibrous mats were dried additionally under reduced pressure for 72 h.

2.2.2. Preparation of Jeff-8Q (Jeff-8Q.Cu^{2+})/onPLA by electrospinning in conjunction with dip-coating (type "on" mats). The complex with a molar ratio of 8Q end groups of Jeff-8Q and Cu^{2+} 1:1 was prepared by mixing 1.0 ml 8 wt% ethanol solution of Jeff-8Q with 1.0 ml 0.85 wt% ethanol solution of $CuCl_2$ for 4 h at room temperature. Jeff-8Q (Jeff-8Q.Cu^{2+})-coated PLA mats (further denoted as Jeff-8Q/onPLA or Jeff-8Q.Cu^{2+}/onPLA) were prepared by immersing the PLA mats in 0.2 wt% ethanol solution of Jeff-8Q or Jeff-8Q.Cu^{2+} for 30 min, and subsequent drying of the mats to constant weight. These fibrous materials were obtained by three repeated dip/dry cycles.

2.3. Preparation of Brb/PMA and Brb/PAA Nps

Brb/PMA (PAA) NPs were obtained by mixing dilute aqueous solutions of Brb and PMA or PAA at

Brb-to-polyacid volume ratios ranging from 1:9 to 9:1 at pH 4.9 ($I = 0.1$). The solution of the bigger volume was added dropwise to the smaller one. In order to form stable aqueous dispersions of NPs from Brb and PMA (PAA), the initial concentration of solutions had to be lowered down to 0.01 mg/mL. The mixing of the solutions was carried out under vigorous stirring at room temperature. The prepared NPs were collected by centrifugation at 9000 rpm for 30 min and washed twice with distilled water. The NPs was redispersed in distilled water using an ultrasonic bath for 30 min.

2.4. Characterization of the electrospun nonwoven textile and Nps

The morphology of the electrospun nonwoven textile was analyzed by scanning electron microscope (SEM, Jeol JSM-5510 (Jeol Ltd., Japan)) after vacuum-coating the samples with gold. The average fiber diameter was determined using Image J software by measuring at least 60 fibers per sample. Static contact angle measurements were carried out using an Easy Drop DSA20E Krűss GmbH apparatus (Germany). A drop of deionized water (10 μL) was deposited on the surface of the mats. The mats were cut at 0° and at 90° with respect to the collector rotation direction. Images for temporal evolution of the contact angle value were taken. The average value of the contact angle was determined based on 20 different measurements for each sample.

The morphology of the redispersed NPs was assessed by transmission electron microscopy (TEM, JEM 2100, JEOL Co. Ltd.). For sample preparation, 2-3 drops of the NPs dispersion was dispensed onto copper grids, which were coated with a carbon film and dried under ambient conditions before examination. The measurement of mean particle size and zeta potential of the prepared in buffer with pH 4.9 NPs was assayed by the dynamic light scattering (DLS). The NPs were analyzed with a NanoBrook 90Plus PALS instrument (Brookhaven Instruments Corporation) equipped with 35 mW red diode laser ($\lambda = 640$ nm) at a scattering angle (θ) of 15°, 25±0.1 °C.

The encapsulation efficiency (%) of Brb in Brb-containing NPs was calculated according to the following equation:

Encapsulation efficiency (%) = actual mass of loaded Brb/initial mass of Brb 100 (1)

2.5. MTT cytotoxicity assay

2.5.1. Assessment of the cytotoxicity of Jeff-8Q- and Jeff-8Q.Cu^{2+}- containing electrospun nonwoven textile by MTT assay.

To evaluate the cytotoxic effect of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile on the proliferation of the HeLa tumor cells, MTT assay was performed [21]. HeLa tumor cells were trypsinized using 0.25% Trypsin–EDTA and counted using a hemocytometer. The cells were seeded in a 96-well microtiter plate at a concentration of 1×10^4 cells/well. After overnight incubation at 37 C in a humid atmosphere containing 5% CO_2 required for cell attachment, the culture medium was replaced and the cells were treated with different types of fibrous mats (Jeff/inPLA, Jeff-8Q/inPLA, Jeff-8Q.Cu^{2+}/inPLA, Jeff-8Q/onPLA, Jeff-8Q.Cu^{2+}/onPLA), with Jeff-8Q or Jeff-8Q.Cu^{2+} (positive control) and cultured only in nutritive medium (negative control) for 72 h. All Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing mats were tested at concentration of 8Q residues 54 g/mL of culture medium. The concentration of 8Q residues in Jeff-8Q and Jeff-8Q.Cu^{2+} was 54 g/mL of culture medium. After culturing on mats, the HeLa cells were washed twice with PBS (pH 7.4), after which 100 μL of MTT solution were added to each well and the cells were incubated at 37 C for 3 h; the supernatants were aspirated and 100 μL of the lysing solution (DMSO:ethanol = 1:1) were added to each well in order to dissolve the obtained formazan. The results from the MTT assay were read using an ELISA plate reader (TECAN, SunriseTM, Grödig/Salzburg, Austria). The absorbance of the dissolved formazan was measured spectrophoto-metrically at a wavelength of 540 nm, ref. 620 nm. Each variant of the fibrous mats was assayed by six measurements.

2.5.2. Assessment of the cytotoxicity of Brb-containing NPs by MTT assay.

HeLa tumor cells and non-tumor BALB/c 3T3 cells (1×10^4 cells/well) in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin were seeded into 96-well flat bottom culture plates for 24 h. After that the cells were washed with fresh medium and were treated with different Brb concentrations (3.1, 6.3, 12.5, 25, 50 and 100 μg/mL) or Brb/PAA and Brb/PMA NPs (the Brb content in the NPs was 3.1, 6.3, 12.5, 25, 50 and 100 μg/mL) for 72 h. HeLa cells or BALB/c

3T3 cells incubated in culture medium only were used as negative control. The further performance of the MTT assay was carried out as described above in 2.5.1.

2.5.3. Percentage of cell viability and selectivity index.

To calculate the percentage of cell viability the following equation was used:

Cell viability (%) = OD_{570} (experimental)/OD_{570} (control) × 100 (2)

Ic_{50} values (i.e. the Brb concentration or the Brb content in the NPs that exerts 50% inhibition with respect to untreated cells) were determined for HeLa and BALB/c 3T3 cells.

The selectivity index (SI) was calculated from the following equation:

Selectivity Index = IC_{50} calculated for non-tumor cells/IC_{50} calculated for tumor cells (3)

2.6. Study of the effect of the fibrous mats and NPs on HeLa tumor cells and on non-tumor BALB/c 3T3 mouse fibroblast cells using dual staining with AO and EtBr

The morphology of the cell nuclei was assessed by double staining with AO and EtBr as described by Wahab et al., 2009 [22]. In brief, the HeLa or BALB/c 3T3 cells were seeded on the various electrospun nonwoven textile (Jeff-8Q/*in*PLA and Jeff-8Q.Cu^{2+}/*in*PLA mats) in 24-well tissue culture plates, in a CO_2 incubator. In the cases of free Brb and Brb/PMA NPs, the cells seeded on sterile glass coverslips placed in 24-well plates were incubated at 37◦C in the presence of various formulations (Brb and Brb/PMA NPs) for 24 h. After 24-h incubation, the fibrous mats or glass cover slips were washed twice with PBS, air-dried at room temperature, stained with AO and EtBr in the ratio of 1:1, and examined by a fluorescence microscope (Leica DM 5000B; Wetzlar, Germany).

2.7. Statistical analysis

The data are given as the mean ± standard deviation (SD). Significance testing was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Nonlinear regression (curve fit) analysis (GraphPad Prism) was applied to determine the concentrations inducing 50% inhibition of the cell growth (IC_{50} values).

3. Results and discussion

3.1. Preparation, morphology and water contact angle of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile

The prepared in the present study Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile of different design is schematically represented on ***Figure 1.*** These materials were obtained by applying one-pot electrospinning (type "*in*") or electrospinning combined with dip-coating (type "*on*").

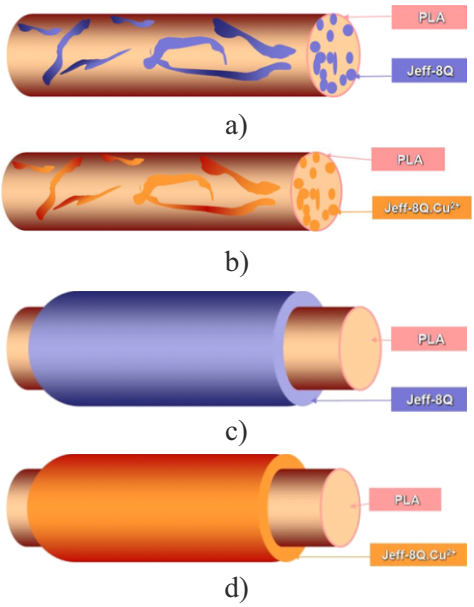
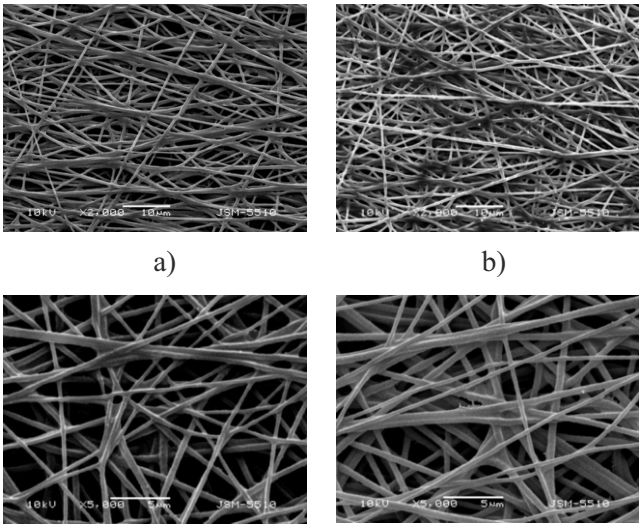


Figure 1 Schematic representation of fibrous materials type "in" prepared by one-pot electrospinning (a,b) and type "on" prepared by electrospinning in conjunction with dip-coating (c,d)

SEM micrographs of obtained Jeff-8Q/*in*PLA, Jeff-8Q.Cu^{2+}/*in*PLA, Jeff-8Q/*on*PLA and Jeff-8Q.Cu^{2+}/*on*PLA fibrous materials are presented in Fig. 2. Neat PLA fibers were defect-free and cylindrical with an average diameter of 600 80 nm. On addition of Jeff-8Q or Jeff-8Q.Cu^{2+} to the PLA spinning solution a decrease in the average fiber diameter was observed (***Figure 2a,b***). The average diameter of Jeff-8Q/*in*PLA mats was 364 87 nm and that of Jeff-8Q.Cu^{2+}/*in*PLA mats was 324 ± 127 nm. Coating the PLA fibers with Jeff-8Q or Jeff-8Q.Cu^{2+} led to increase in the fiber diameter (Fig. 2c,d). The average fiber diameter values were 650 110 nm and 700 170 nm for Jeff-8Q/*on*PLA and Jeff-8Q.Cu^{2+}/*on*PLA mats, respectively.



a) b) c) d)

Figure 2 SEM micrographs of electrospun mats of: Jeff-8Q/inPLA (a), Jeff-8Q.Cu^{2+}/inPLA (b), Jeff-8Q/onPLA (c) and Jeff-8Q.Cu^{2+}/onPLA (d). Magnification ×2000 (a and b) and ×5000 (c and d).

A water contact angle of 126.8 ± 2.3° was determined for the neat PLA mats (***Figure 3a***). The presence of Jeff-8Q or its complex with Cu^{2+} led to hydrophilization of the fibrous materials. It is interesting to note that in the case of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing mats (both types "*in*" and "*on*") the water droplet had an oval shape (***Figure 3c***) or the wetted area had an elliptical shape (Fig. 3b,d,e) as compared to the spherical one of the droplet observed for PLA mats (***Figure 3a***). It seems that the water droplet tends to elongate along the direction of fiber alignment [23]. The experimentally determined values of the water contact angle were q_x=27.4 ± 2.5°, q_y=49.8 ± 4.4°, and Δq=22.4° (where q_x was the water contact angle measured in the x-direction, e.g. in the direction of the collector rotation, q_y was the water contact angle measured in the y-direction, e.g. at 90° with respect to the collector rotation direction and Δq=q_x-q_y was the degree of wetting anisotropy) for type "*in*" Jeff-8Q-containing mat, q_x=q_y=0° for type "*on*" Jeff-8Q-containing mat and q_x=q_y=0° for Jeff-8Q.Cu^{2+}-containing mats (both types "*in*" and "*on*"), respectively (***Figure 3b-e***).

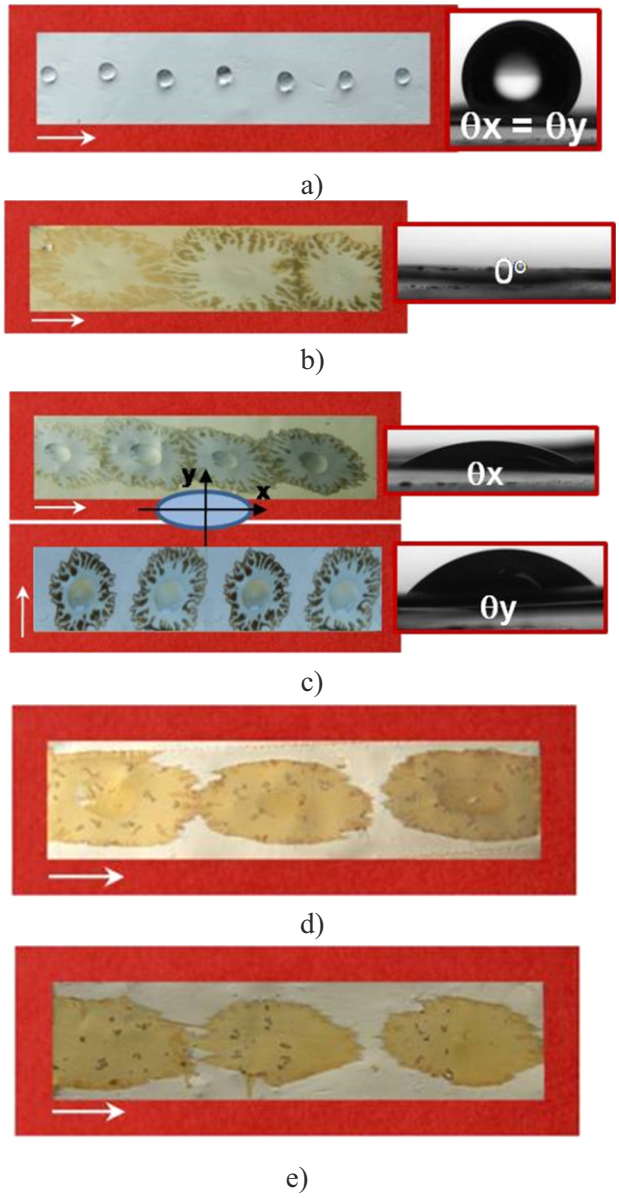


Figure 3 Digital images of water droplets deposited on the surfaces of electrospun nonwoven textile from (a) PLA, (b) Jeff-8Q/onPLA, (c) Jeff-8Q/inPLA, (d) Jeff-8Q.Cu^{2+}/inPLA, and (e) Jeff-8Q.Cu^{2+}/onPLA. The direction of the collector rotation is indicated by an arrow. q_x and q_y was measured at 0° and 90° with respect to the collector rotation direction, espectively. Different sections of the digital images of water droplets represent different wetting anisotropy (c).

3.2. Preparation and morphology of Brb-containing NPs

The Brb molecule has a quaternary ammonium group (***Figure 4a***) and this provides a favorable possibility of ionic interaction with suitable polymeric carriers. Suitable carriers may be ionogenic biocompatible polymers, for example synthetic polyacids such as PAA or PMA. In the present study, stable aqueous dispersions of NPs based on complexes of Brb and PMA or PAA, were successfully prepared by vigorously mixing their

dilute buffered aqueous solutions (0.01 wt%) of pH 4.9 at various mole fractions of Brb. The preparation of these NPs is schematically represented in ***Figure 4b***.

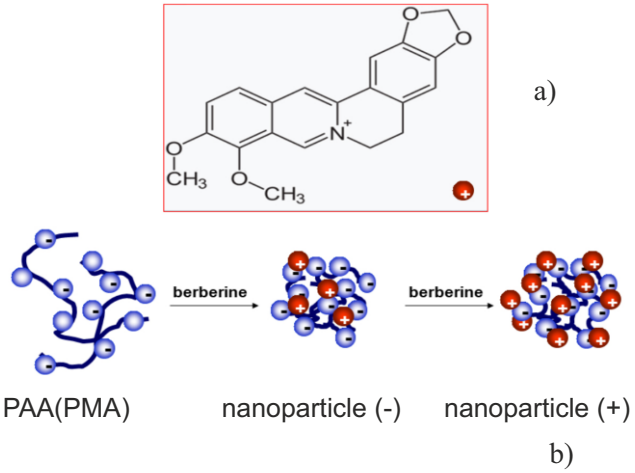


Figure 4 (a) Structure of Brb, (b) Schematic representation of the preparation of NPs based on complex between Brb and PAA or PMA at pH 4.9.

The morphology of the Brb-containing NPs was determined by TEM. As seen in ***Figure 5a***, the Brb/PMA (PAA) NPs were of spherical shape. The dependence of the mean diameter of NPs for the pair Brb/PMA and Brb/PAA on the Brb mole fraction at partner concentration of 0.01 mg/mL was studied by DLS (***Figure 5b***). As seen, the mean diameter increased on increasing the mole fraction of Brb and passed through a maximum at values of mole fraction of Brb at which maximum amount of complexes was obtained at pH 4.9. Monomodal distribution was obtained whatever the value of the mole fraction of Brb was. It was found that the zeta potential of the Brb/PMA and Brb/PAA NPs was significantly dependent on the Brb mole fraction and became positive at mole fraction of Brb ≥0.68 and ≥0.64 for Brb/PMA and Brb/PAA, respectively. The decrease in the negative value of the zeta potential of the NPs on increasing the mole fraction of Brb were probably due to the presence of smaller amount of free carboxylate groups on the NP surface, because of the interaction between these carboxylate groups and the quaternary ammonium groups of Brb (***Figure 4b***). The amount of free quaternary ammonium groups of Brb on the NP surface increased at a mole fraction of Brb ≥0.68 and ≥0.64 for the Brb/PMA and Brb/PAA pair, respectively.

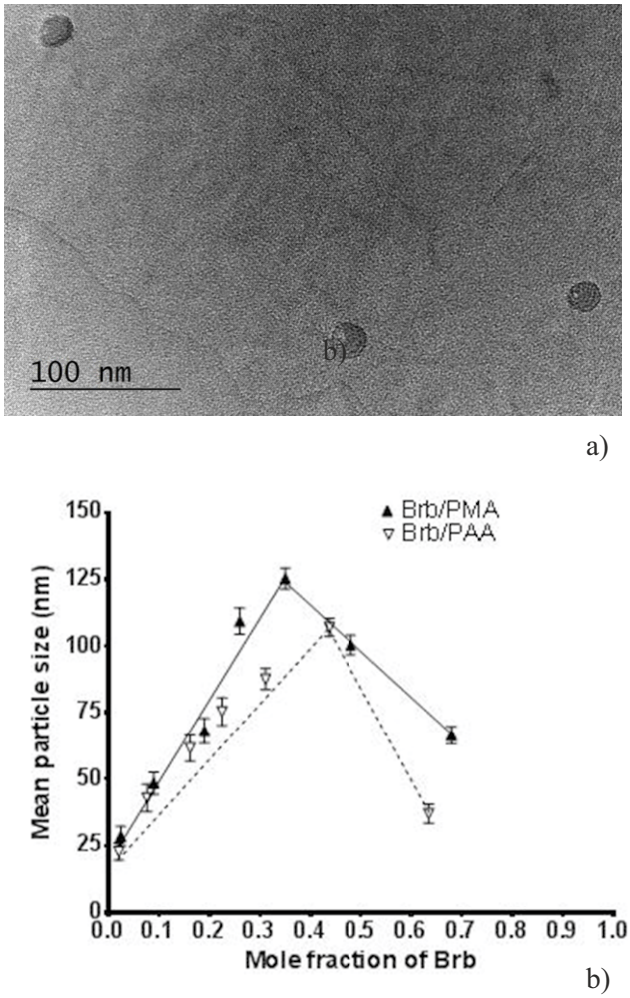


Figure 5 TEM micrographs of Brb/PMA NPs (mole fraction of Brb of 0.35) (a). Dependence of the mean size (b) on the mole fraction of Brb for Brb/PMA and Brb/PAA NPs at partner concentration of 0.01 wt %; pH 4.9; I = 0.1, 25±0.1°C.

In the case of Brb/PMA and Brb/PAA NPs the encapsulation efficiency was observed to approach a maximum value of 58.9 ± 0.5% and of 78.4 ± 0.9 %, respectively, at values of Brb mole fraction at which maximum amount of complexes was obtained.

3.3. Cytotoxicity of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile and Brb-containing NPs against HeLa tumor cells and non-tumor BALB/c 3T3 fibroblast cells

3.3.1. Cytotoxicity of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile. In the present study, the cytotoxic effect of the obtained PLA mats containing Jeff-8Q or its Cu^{2+}

complexes against HeLa tumor cells was evaluated using the MTT assay. As seen in Figure 6, after 72 h of incubation, the greatest decrease of the proliferative activity of the HeLa cells was observed in the case of Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing mats (both types "*in*" and "*on*"). The percentage of viable HeLa cells for the Jeff-8Q (Jeff-8Q.Cu^{2+})-containing mats was close to that of the free Jeff-8Q (Jeff-8Q.Cu^{2+}). In contrast, the Jeff/in PLA mat lead to a statistically insignificant ($p > 0.05$) decrease in the cell viability as compared to untreated HeLa cells.

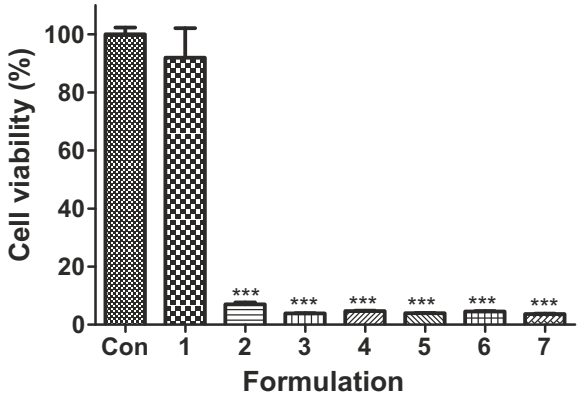
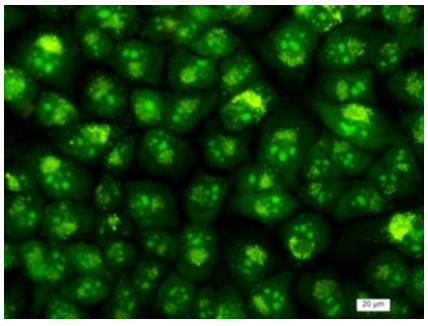
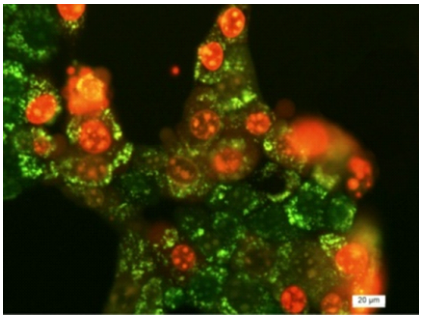


Figure 6 Cell viability of HeLa cell line tested by MTT method after 72 h for incubation with different formulations: untreated HeLa cells (Con); Jeff/*in*PLA mat (1), Jeff-8Q/*in*PLA mat (2), Jeff-8Q.Cu^{2+}/*in*PLA mat (3), Jeff-8Q/*on*PLA mat (4), Jeff-8Q.Cu^{2+}/*on*PLA mat (5), Jeff-8Q (6), Jeff-8Q.Cu^{2+} (7). ***$p<0.001$.



a)



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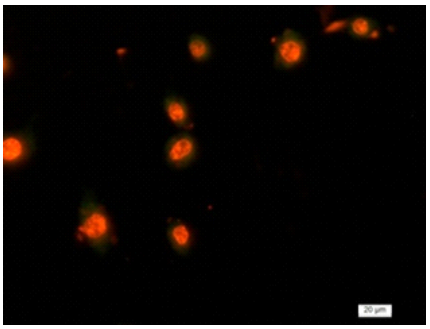
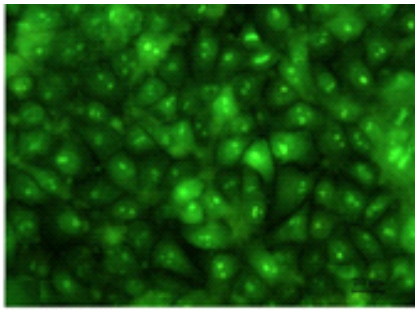
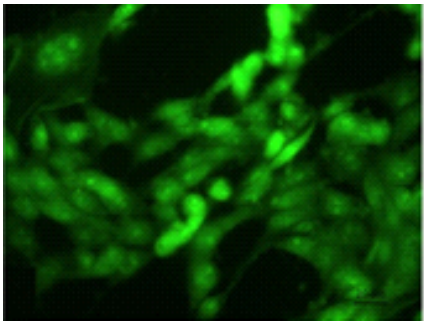


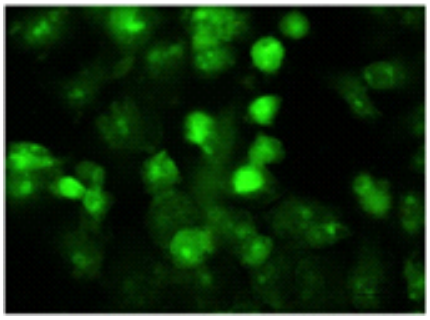
c)



d)



e)



f)

Figure 7 Fluorescence micrographs of AO and EtBr double-stained HeLa tumor cells (a-c) and non-tumor BALB/c 3T3 mouse fibroblast cells (d-f) incubated for 24h. Non-treated HeLa cells (a) and HeLa cells after incubation with: (b) Jeff-8Q/inPLA mat, (c) Jeff-8Q.Cu^{2+}/inPLA mat; non-treated non-tumor BALB/c 3T3 mouse fibroblast cells (d) and BALB/c 3T3 cells after incubation with: (e) Jeff-8Q/inPLA mat, (f) Jeff-8Q.Cu^{2+}/inPLA mat, scale bar = 20 μm.

In order to detect the morphological changes in the HeLa tumor cells and non-tumor BALB/c 3T3 mouse fibroblast cells occurring at the 24th h after contact with the fibrous mats, the method of intravital double staining with fluorescent dyes (AO and EtBr) was applied (***Figure 7***). The untreated HeLa cells are characterized by a normal morphological structure - pale green nuclei and bright yellow-green nucleoli, accumulation of orange granules in the perinuclear region (***Figure 7a***). In contrast to them, upon cultivation of the HeLa cells on a Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing mats, a different degree of cell destruction was observed (***Figure 7 b,c***) - light-green, yellow-orange, orange-red stained dead cells, the majority of which have the morphological characteristics of early or late apoptosis.

As seen in Figure 7d, the non-treated non-tumor BALB/c 3T3 mouse fibroblast cells showed normal morphology and monolayer growth. Upon culturing of the non-tumor BALB/c 3T3 mouse fibroblast cells on mats containing Jeff-8Q or its complex with Cu^{2+} the cells remained green-colored, but had a sparse monolayer growth and were of different shape (elongated, rounded) and size (***Figure 7e,f***). Cells with unevenly distributed chromatin in the form of dense green areas were observed. The results indicated that Jeff-8Q (Jeff-8Q.Cu^{2+})-containing mats exhibited a higher cytotoxicity against HeLa tumor cell than against non-tumor BALB/c 3T3 mouse fibroblast cells.

3.3.2. *Cytotoxicity of Brb-containing Nps.*

As seen in Figure 7d, the non-treated non-tumor BALB/c 3T3 mouse fibroblast cells showed normal morphology and monolayer growth. Upon culturing of the non-tumor BALB/c 3T3 mouse fibroblast cells on mats containing Jeff-8Q or its complex with Cu^{2+} the cells remained green-colored, but had a sparse monolayer growth and were of different shape (elongated, rounded) and size (***Figure 7e,f***). Cells with unevenly distributed chromatin in the form of dense green areas were observed. The results indicated that Jeff-8Q (Jeff-8Q.Cu^{2+})-containing mats exhibited a higher cytotoxicity against HeLa tumor cell than against non-tumor BALB/c 3T3 mouse fibroblast cells.

3.3.2. *Cytotoxicity of Brb-containing Nps.*

The cytotoxic effect of the prepared Brb-containing NPs against HeLa tumor cells and non-tumor BALB/c 3T3 mouse fibroblast cells was assessed by a MTT test. After 72 h of incubation, the percentage of viable HeLa cells in the presence of Brb/PAA NPs decreased to 50.4 ± 7.6, 16.8 ± 3.3, and 8.8 ± 0.4% at Brb concentrations in NPs 25, 50 and 100 μg/mL, respectively (***Figure 8b***). In this case the observed cytotoxicity was similar to that of free Brb (***Figure 8a,b***). The most significant decrease in the proliferative activity of HeLa cells was detected at 72nd h of incubation in the presence of Brb/PMA NPs – the cell viability was reduced to 20.8 ± 1.1, 18.0 ± 3.1 and 8.7 ± 0.3% at Brb concentrations in NPs 25, 50 and 100 μg/mL, respectively (***Figure 8c***). The IC_{50} value for free Brb, which is the concentration required for 50% cell growth inhibition, was determined to be 24.91 μg/mL after 72 h of incubation. IC_{50} value for Brb/PAA NPs after 72 h of incubation was 24.51 μg/mL and for Brb/PMA NPs - 2.85 μg/mL, respectively. The obtained results showed that Brb-containing NPs exerted a significant cytotoxicity against HeLa tumor cells, which depended on the concentration of Brb in the NPs.

As seen in Fig. 8 d, the viability of the BALB/c 3T3 cells was substantially reduced after treating them with free Brb when its concentration was equal to or higher than 25 g/mL ($p<0.001$). The percentage of viable BALB/c 3T3 cells in the presence of Brb at concentrations 25, 50 and 100 g/mL decreased to 43.2 ± 1.7%, 24.9% ± 1.5%, and 12.9 ± 0.7%, respectively. After 72 h of incubation, Brb-containing NPs exhibited a lower cytotoxicity to the non-tumor BALB/c 3T3 cells than an equal concentration of free Brb (***Figure 8 d-f***). The BALB/c 3T3 cell viability was reduced to 77.50 ± 0.8%, 74.2 ± 1.0% and 71.7 ± 0.6% for Brb/PAA NPs at Brb concentrations in NPs 25, 50 and 100 μg/mL respectively. The viability of cells was 82.0 ± 2.0%, 76.7% ± 3.1%, and 72.0 ± 1.5% for Brb/PMA NPs at Brb concentrations in NPs 25, 50 and 100 μg/mL, respectively. The IC_{50} value for Brb was calculated to be 16.89 μg/mL after 72 h of incubation. IC_{50} value for Brb/PAA NPs after 72 h of incubation was 311.1 μg/mL μg/mL and for Brb/PMA NPs - 237.5 μg/mL, respectively. The selectivity index (SI), which indicates the cytotoxic selectivity for Brb-containing NPs and free Brb against cancer cells versus normal cells was calculated. This index for free Brb was calculated to be 0.7, for Brb/PAA NPs was 12.7 and

for Brb/PMA NPs - 83.3, respectively. The obtained SI values for Brb-containing NPs revealed that these NPs exhibited a high degree of selectivity in HeLa tumor cells. The Brb/PMA (PAA) NPs exerted higher cytotoxicity against HeLa tumor cell than non-tumor BALB/c 3T3 mouse fibroblast cells. The SI value for Brb indicated that this compound was significantly cytotoxic to the HeLa tumor cells as well as to the non-tumor BALB/c 3T3 mouse fibroblast cells.

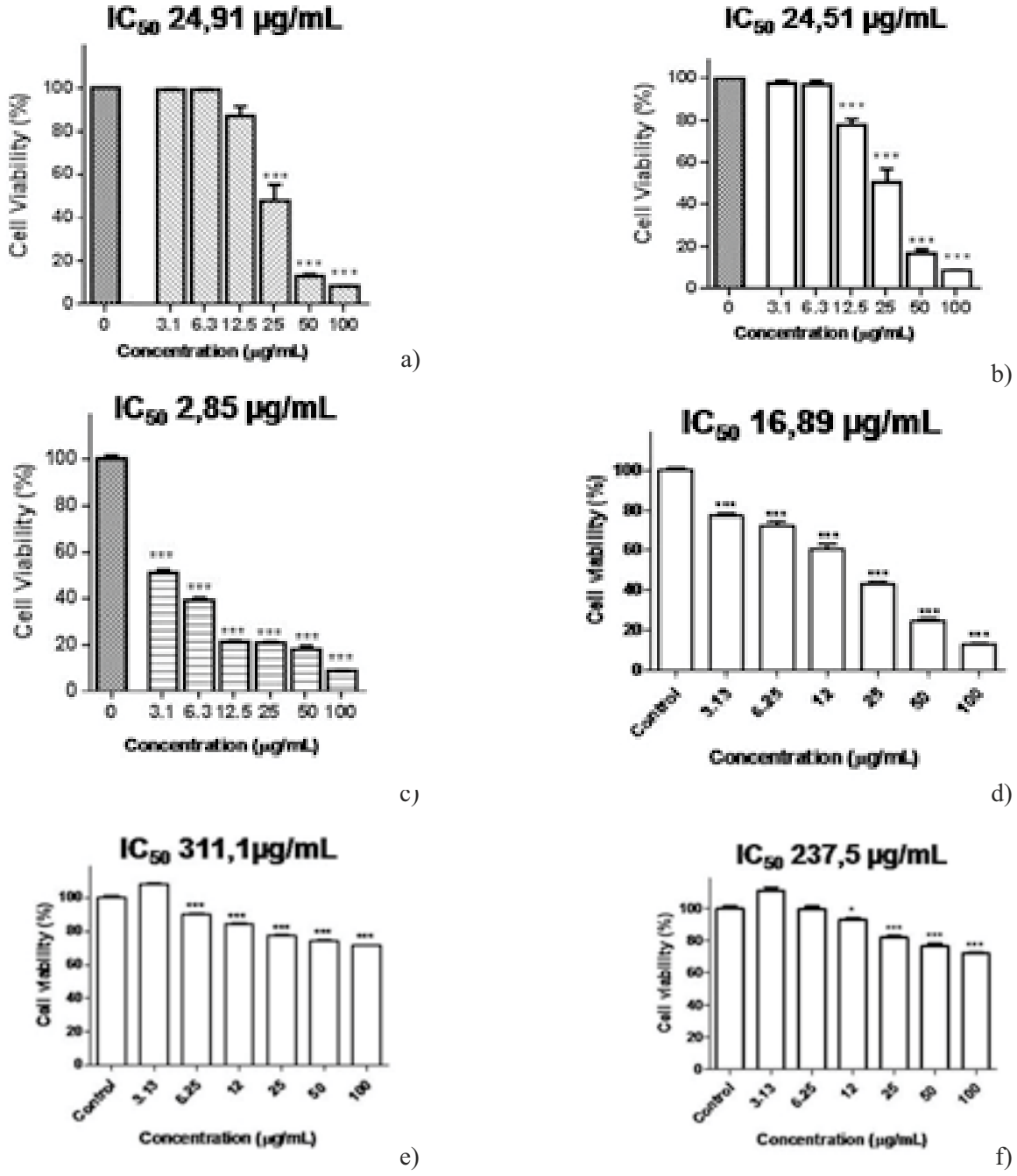


Figure 8 Effect of free Brb (a,d), Brb/PAA NPs (b,e) and Brb/PMA NPs (c,f) on the viability of the HeLa tumor cells (a-c) and non-tumor BALB/c 3T3 mouse fibroblast cells (d-f) incubated 72 h in the presence of Brb in the tested compounds *in vitro*. Non-treated HeLa cells (0) or BALB/c 3T3 fibroblast cells – control. Data are mean ± SD of six replicates. ***p<0.001, **p<0.01, *p<0.05.

Experiments were carried out aimed to clarify whether inhibition of proliferation of HeLa tumor cells takes place through apoptosis. For this purpose HeLa cells were incubated for 24 h in the presence of Brb/PAA and Brb/PMA NPs or free Brb and then stained with AO and EtBr. Morphological changes in the HeLa tumor cells incubated in the presence of Brb-containing NPs or free Brb and processed with AO and EtBr were observed by fluorescence microscope (Fig. 9b.c). After staining the non-treated HeLa cells, they had normal morphology - predominantly pale green nuclei and bright yellow-green nucleoli were observed (Fig. 9a). Accumulations of orange granules located perinuclearly were also found. As shown in Fig. 9b,c, cell blebbing, nuclei with condensation and aggregation of chromatin, fragmentation of the nuclei and appearance of apoptotic bodies (a typical sign of apoptosis) were observed in HeLa cells that had been in contact with free Brb or Brb/PMA NPs. In these cases the cell monolayer was damaged.

Morphological changes in the non-tumor BALB/c 3T3 mouse fibroblast cells treated with Brb-containing NPs or with free Brb and stained with AO and EtBr were also analyzed by fluorescence microscope (***Figure 9 f,e***). The non-treated BALB/c 3T3 cells showed normal morphology and monolayer growth (***Figure 9d***). In contrast to them, BALB/c 3T3 cells treated with free Brb showed sparse monolayer growth and had significant morphological changes characteristic of apoptosis (***Figure 9f***). The cells were round, green-stained, with unevenly distributed chromatin. In this case, cells with condensation and aggregation of chromatin, and fragmentation of the nuclei were observed.

In contrast to them, Brb-containing NPs induced only slight changes in the cell morphology. No significant changes in the staining of nuclei and cytoplasm were observed compared to control untreated cells. The cells were slightly enlarged and had monolayer growth. Only single cells with unevenly distributed chromatin were observed. Cell nuclei at different stages of mitosis were also observed. The performed studies have shown that Brb-containing NPs caused more significant changes in the growth and morphology of the HeLa tumor cells as compared to that of the non-tumor BALB/c 3T3 mouse fibroblast cells. The obtained results are consistent with the data from the MTT assay and support the statement that NPs containing Brb induce death of HeLa tumor cells through apoptosis.

4. Conclusions

In the present study, for the first time polymeric nanosized materials (electrospun nonwoven textile and NPs) containing 8-hydroxyquinoline derivative (Jeff-8Q), its complex with Cu^{2+} or Brb were successfully prepared. The incorporation of these bioactive compounds in the nanosized materials impart to the materials good *in vitro* antitumor activity against HeLa tumor cells. The induction of apoptosis is one of the major mechanisms of the antitumor activity of the prepared nanosized materials, which is verified by the performed fluorescence microscopy analyses. The cytotoxicity of the Jeff-8Q- and Jeff-8Q.Cu^{2+}-containing electrospun nonwoven textile and Brb-containing NPs against HeLa tumor cells was considerably higher as compared to that against non-tumor BALB/c 3T3 mouse fibroblast cells. These results indicate that the obtained nanosized materials have the potential to be used as novel materials for treatment of cervical tumors.

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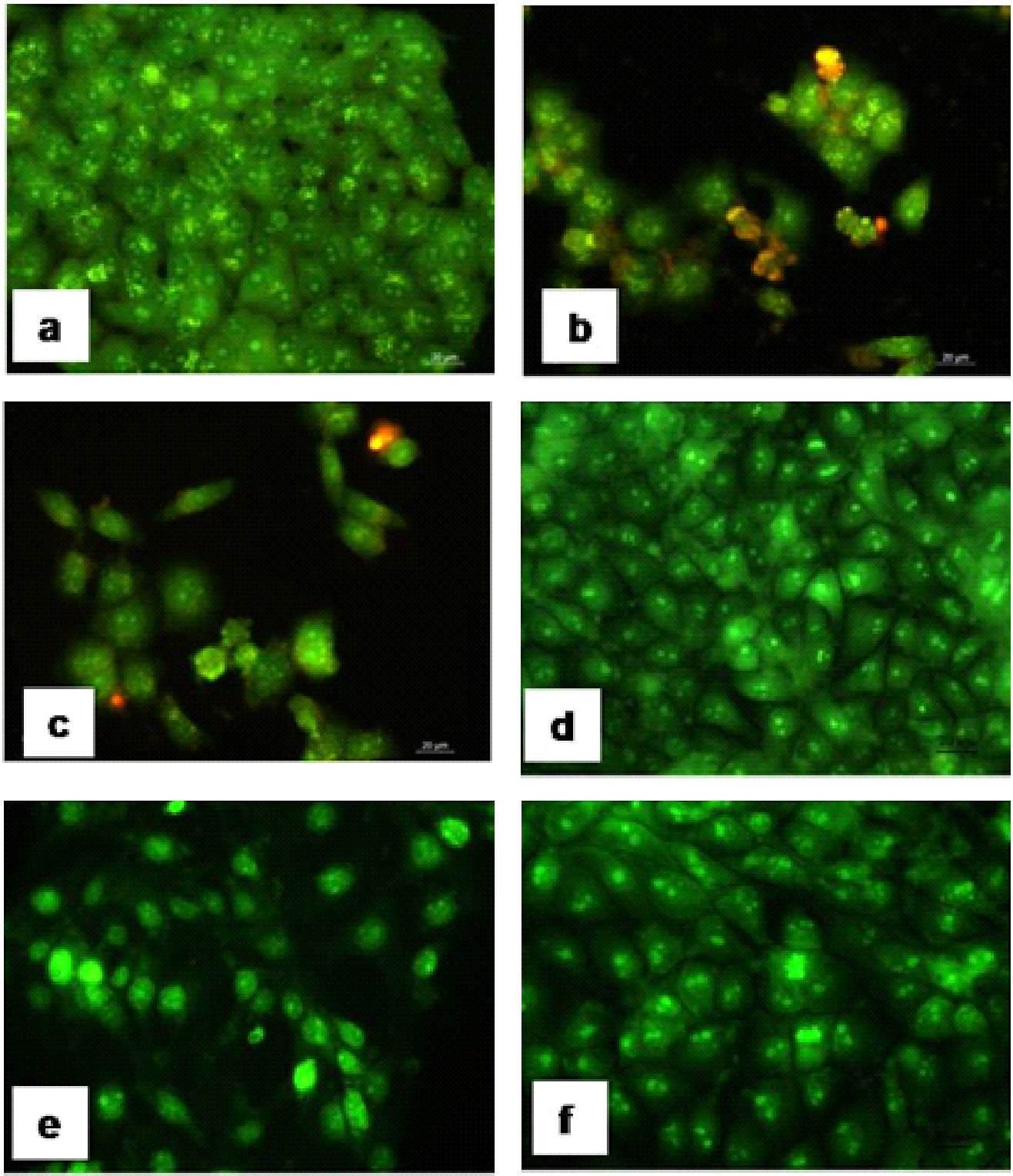

Figure 9 Fluorescence micrographs of AO and EtBr double-stained HeLa tumor cells (a-c) and non-tumor BALB/c 3T3 mouse fibroblast cells (d-f) incubated for 24 h.
(a) Non-treated HeLa cells (control), and HeLa cells after incubation with:
(b) aqueous solution of Brb,
© Brb/PMA Nps, non-treated non-tumor BALB/c 3T3 mouse fibroblast cells
(d) and BALB/c 3T3 cells after incubation with:
(e) aqueous solution of Brb,
(f) Brb/PMA NPs, scale bar = 20 μm.

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