Novel Nanocomposite of Nano Fe₃O₄ and Polylactide Nanofibers for Application in Drug Uptake and Induction of Cell Death of Leukemia Cancer Cells

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Novel nanocomposites of polylactide (PLA) nanofibers and tetraheptylammonium-capped Fe₃O₄ magnetic nanoparticles have been prepared and utilized to realize the efficient accumulation of anticancer drug daunorubicin in target cancer cells. The observations of optical microscopy and confocal fluorescence microscopy indicate that the PLA nanofibers and Fe₃O₄ nanoparticles may contribute to their beneficial effects on intracellular drug uptake of leukemia K562 cell lines in which the efficiently enhanced accumulation of anticancer drug daunorubicin on the membrane of cancer cells could be observed. Meanwhile, the electrochemical detection and the microculture tetrazolium studies were also explored to probe the effect of the relevant nanomaterials on the drug uptake of cancer cells. The results illustrate that the nanocomposites could effectively facilitate the interaction of daunorubicin with leukemia cells and remarkably enhance the permeation and drug uptake of anticancer agents in the cancer cells, which could readily lead to the induction of the cell death of leukemia cells. This observation suggests a new perspective for the targeted therapeutic approaches of cancers.

Introduction

The efficient cure of cancers is still an important and critical challenge in disease treatments. The conventional methods of chemotherapy or radiotherapy to cancer cells suffer from a number of disadvantages, particularly involving efficacy and safety issues. Relative research studies have shown that the treatment efficiency of cancer chemotherapy depends not only on the anticancer drug itself but also on how it is delivered to targets.1 In the clinical cancer therapies, the multidrug resistance is still a major obstacle and usually correlates with the failure of chemotherapy, which will result in the loss of the drugs uptake and the low drug concentration in the target.

Nanotechnology offers efficient alternatives for the respective cancer diagnostics and tumor target treatment due to the unique properties of nanomaterials, such as large surface-to-volume ratio, porous structure, embedded effect, and size effect, which have been well-recognized to offer potential promising applications in biomedical engineering. Now, much effort has been extended to the development of novel nanocomposites and biomaterials for DNA detections,2 intracellular labeling,3 drug carrier,4 cancer targeting,5 imaging,6 etc. Recent reports have demonstrated that the drug-coated polymer nanospheres and nanoparticles could efficiently increase the amount of intracellular anticancer drug, where much attention has focused on the research of the biocompatible and biodegradable polymers or nanoparticles due to their promising biomedical applications.7–18 At the same time, the capability of anticancer drugs against biotransformation and rapid clearance from the body could be facilitated by a combination with nanospheres or nanoparticles, which could further afford the proper biodistribution of anticancer drugs to target tumor cells and tissues. Hence, it is promising that the association of antitumor drugs and nanomaterials could be utilized to overcome the noncellular and cellular based mechanisms of drug resistance and to increase the selectivity of drugs toward cancer cells while reducing their toxicity toward normal tissues.

As is known, cancer cells execute their malicious activities through the cell membranes that screen and transmit stimulatory and inhibitory signals into the cells and out of them. Meanwhile, they can produce and secrete substances found inside of cells, including carcinogenic antigens, hormones, metabolites, growth factors, enzymes, and so on, which result in the alterations of cancerous tissues, enzymes, proteins, and other growth factors. In this regard, the nanomaterials with effective drug carriers are expected to overcome the noncellular and cellular based mechanisms of drug resistance and to increase the selectivity of drugs toward cancer cells while reducing their toxicity toward normal tissues.

biologic properties. Among those, the membrane proteins on the cancer cell interface, such as P-glycoprotein, play oppositional roles in the chemotherapy process. However, these proteins could be influenced by the nanomaterials that were modified with special active groups. Such surface groups containing unique interfacial structure and chemical properties can execute a significant influence on the transportation of drugs through the cell membrane. On the basis of the layer-by-layer (LBL) technique, the polymeric shells, known as “nanocapsules”, can exhibit good performance with regard to changes in pH, solvent, and temperature by alternating adsorption of the oppositely charged species onto a charged template (core).

Polylactic acid polymer, namely, polylactide (PLA), is one kind of biocompatible and biodegradable material. It undergoes scission in the body to monomeric units of lactic acid, which is a natural intermediate in the carbohydrate metabolism. These characteristics make them well suitable for applications in resorbent sutures, in readily adopted drug release, and as implants for orthopaedic surgeries or blood vessels. It shows good perspective as potential material for the replacement of the body’s tissue. Based on these properties, in this work, the PLA nanofibers that are fabricated by electrospinning techniques have been applied in the research of controllable drug delivery of cancer cells. We demonstrate the possibility of extending the nanofibers to the fields of the enhanced adsorption of anticancer drugs in target cancer cells.

Considering the unique properties, i.e., good biocompatibility, super-paramagnetic property, low toxicity, and easy preparation of the Fe3O4 nanoparticles,20-24 we have further explored the feasibility to combine the tetraethylammonium-capped Fe3O4 nanoparticles (ca. 30 nm) with the target drug uptake of anticancer agents to realize the controllable targeting of the PLA and drug. Through the modification of the magnetic Fe3O4 nanoparticles that were produced by the electrochemical deposition under oxidizing conditions (EDOC),23,25 anticancer drugs could be readily assembled on the surface of the magnetic nanoparticles and fixed at the abnormal tissue by applying an external magnetic field. The results demonstrate the possibility of utilizing the respective magnetic nanoparticles to enhance the local drug concentration in the target tumor cells and hence the efficiency of the cancer treatments. Moreover, since iron oxide could be phagocytosed by cancer cells and subsequently interacted with the relevant components inside the cells, the magnetic nanoparticles could offer promising potential for early diagnostic and targeted therapeutic approaches of cancers.

**Experimental Details**

**Apparatus.** Scanning electron micrographs were obtained with a scanning electron microscope (SEM) (Hitachi, S-3000N) by using the relevant components inside the cells, the magnetic nanoparticles could offer promising potential for early diagnostic and targeted therapeutic approaches of cancers.

was used to capture the optical microscope images of the target cells under experimental conditions identical to those in the confocal fluorescence microscopic studies. Electrochemical detections were performed on a CHI660b electrochemistry workstation (CHI Inc., USA).

**Materials.** Ultrapure water was prepared by distillation and then purified with a Milli-Q purification system (Millipore Trading Co., Ltd.) to a specific resistance of $>18 \ \text{M\Omega}\ \text{cm}$ at 25 °C. Tetraethylammonium 2-propanol, high-purity Fe sheets, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid sodium] (HEPES), penicillin, streptomycin, and microculture tetrazolium (MTT) were purchased from Sigma. The bulk PLA was purchased from Maikang in Jinan, China. The chloroform, NaHCO3, aluminum foil, and dimethyl sulfoxide (DMSO) were purchased from Sinoreagent. The RPMI 1640 medium was purchased from GIBCO. The fetal calf serum (FCS) was purchased from Hyclone, USA. The daunorubicin (DNR) was purchased from Aldrich. All the reagents were analytical grade.

**Preparation of Fe3O4 Nanoparticles.** Fe3O4 nanoparticles were prepared by the EDOC method.25 Generally speaking, the electrolysis processes were carried out in a 0.1 mol/L tetraethylammonium 2-propanol solution using the anode of high-purity Fe sheets and cathode of glassy carbon. A current density of $10-40 \ \text{mA/cm}^2$ was applied for electrolysis. The deposited clusters were capped with tetraethylammonium, which acted as a stabilizer of the colloidal nanocrystallites. Following deposition, a hydrothermal treatment was taken to improve the composition and structure. TEM characterization has indicated that the average size of the Fe3O4 magnetic nanoparticles is about 30 nm (see Supporting Information) and the as-synthesized magnetic nanoparticles possess an even size distribution. The synthesized Fe3O4 nanoparticles were suspended in ultrapure water at the concentration of $2.3 \times 10^{-4} \ \text{mg/mL}$.

**Preparation of PLA Nanofibers.** PLA nanofibers were fabricated by electrospinning techniques. First, bulk PLA (Mw = 340000) was dissolved in the solvent of chloroform (10 wt%) and stirred for 2 h. Then it was loaded into a syringe with a stainless steel needle (500 μm in diameter) and connected with the anode of voltage. Meanwhile, the collecting equipment of aluminum foil was connected with the cathode of voltage. A voltage of 10 kV was applied between the needle and the aluminum foil. The distance from the tip of the needle to the aluminum foil is 10 cm and the feeding is 1 ml/h.

**Cell Culture.** Leukemia K562 cells were cultured in a flask in a RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO2.

**Confocal Fluorescence Microscopic Studies.** DNR (7.5 × 10−5 mol/L) together with Fe3O4 nanoparticles (2.3 × 10−4 mg/mL) or/and PLA nanofibers (2.5 × 10−4 mg/mL) were injected into a cell culture for incubation. In the control experiment, only DNR (7.5 × 10−5 mol/L) or nanocomposite solution was injected into the cell culture for incubation under identical experimental conditions. The culture was detected after 1 h of incubation. Then the freshly prepared sample was dropped on a clean slide immediately before the measurement. Relative fluorescence microscopic studies were carried out by using a confocal fluorescence microscope. The excitation and emission fluorescence wavelengths of the DNR were 480 and 560 nm, respectively (see Supporting Information). All measurements were carried out at ambient temperature (20 ± 2 °C).

**Electrochemical Investigation.** After incubation of the targeted cells with Fe3O4 nanoparticles or/and PLA for 1 h, a 1.0 mL medium was centrifuged at 1000g for 10 min; 0.5 mL of supernatant was transferred to an electrolytic cell containing 5.0 mL of 0.1 M phosphate buffer solution (pH 7.2). Differential pulse voltammetry was used to detect the electrochemical response of DNR under different conditions. All measurements were carried out at ambient temperature in a three-component electrochemical cell consisting of a glassy carbon electrode as the working electrode, a Pt wire as the counter electrode, and an Ag wire as the reference electrode. During electrochemical measurements, the blending solution of Fe3O4 nanoparticles (2.3 × 10−4 mg/mL) and PLA nanofibers (2.5 × 10−4 mg/mL) was added to the relative solutions including DNR (2.5 × 10−4 mg/mL) and PLA nanofibers (2.5 × 10−4 mg/mL) was injected into the cell culture for incubation. In the control experiment, only DNR (7.5 × 10−5 mol/L) or nanocomposite solution was injected into the cell culture for incubation under identical experimental conditions. The culture was detected after 1 h of incubation. Then the freshly prepared sample was dropped on a clean slide immediately before the measurement. Relative fluorescence microscopic studies were carried out by using a confocal fluorescence microscope. The excitation and emission fluorescence wavelengths of the DNR were 480 and 560 nm, respectively (see Supporting Information). All measurements were carried out at ambient temperature (20 ± 2 °C).
10^(-4) mol/L). In the control experiments, only the solution of Fe_3O_4 nanoparticles (2.3 × 10^(-4) mg/mL) or PLA nanofibers (2.5 × 10^(-4) mg/mL) was injected into the solutions of DNR.

**MTT Assays.** The inhibition of cell growth was measured by a MTT assay. Briefly, K562 cells in the log phase were seeded in a 96-well plate at a concentration of 1.0 × 10^4 cells per well. The cells were cultured in a RPMI medium 1640 with 100 μg/mL streptomycin and 100 μg/mL penicillin in an incubator with 3 mM HEPES and supplemented with 10% fetal calf serum. MTT assays on human sensitive leukemia cell line K562 were performed under different concentration ratios between the Fe_3O_4 magnetic nanoparticles (2.5 × 10^(-4) mg/mL) and PLA nanofibers (2.5 × 10^(-4) mg/mL), respectively. Each experiment was repeated at least four times. Controls were cultivated under the same conditions without addition of DNR and/or the nanocomposites. Each culture was incubated at 37 °C for 72 h in a 95% air and 5% CO_2 incubator, and then, 20 μL of 5 mg/mL MTT was added into the wells and further incubated for an additional 4 h. Subsequently, it was centrifuged at 1000 rpm for 10 min and the supernatant was discarded, followed by addition of 150 μL of DMSO into each well and incubation in the shaker incubator at 37 °C with a gentle shake. Then the optical density (OD) was read at a wavelength of 540 nm. Relative inhibition of cell growth was expressed as follows: % = (1 – [OD]_test/[OD]_control) × 100.

**Results and Discussion**

**Characterization and Dissolution of PLA Nanofibers.** The SEM was utilized to characterize the synthesized PLA nanofibers. Figure 1 illustrates the relative SEM image of the PLA nanofibers (magnification folds: 10000×). As learned from the photograph, the nanofibers exhibit a uniform morphology, with an estimated size of 100 nm in width. The nanofibers usually show a length of several micrometers and can be scissored in the ultrasonic treatment.

In the study of the effect of PLA on the interaction between the DNR and leukemia K562 cells, the PLA solution was freshly prepared in ultrapure water with ultrasonic treatment. The concentration of the stock solution of PLA colloidal suspension solution is 2.5 × 10^(-4) mg/mL. Then the PLA nanofibers were ultimately broken up by the ultrasonic method for 1 min and the size of the PLA fragments was at the micrometer level.

**Microscopic Studies of the Enhanced Cellular Uptake of DNR.** The optics microscopic method and the laser confocal microscopic method were utilized to characterize the samples of different effects of nanostructures on the cells after 1 h of incubation. The controlled experiments were performed to reveal the binding effect of PLA nanofibers or/and Fe_3O_4 magnetic nanoparticles on the interaction between the leukemia K562 cells and DNR.

The optic microscopy images of the interaction between the leukemia K562 cells and the anticancer drug DNR are shown in Figure 2. The panoramic images of the cancer cells are recorded in the presence of DNR only (Figure 2b), PLA nanofibers only (Figure 2c), and DNR together with PLA nanofibers (Figure 2d), respectively. Initially, the microscopic studies demonstrated that there existed some distinguishable differences on the extra cellular membrane for the typical image of the individual leukemia cell upon treatment of PLA nanofibers, compared with the untreated cells shown in Figure 2a. Similar differences also appeared after DNR and PLA nanofibers were simultaneously introduced in the cell system. The prevalent death of cancer cells in the optical sights indicated that the injection of the nanofibers obviously facilitated the absorption of DNR into the leukemia cells (Figure 2d) since the respective treatment of DNR to these cancer cells exhibited poor efficiency (Figure 2b). It thus suggested that the biologically active PLA not only interacted with the leukemia K562 cells by itself but also exerted some positive impact on the interaction between the DNR and leukemia K562 cells. The detailed reason is still unknown and needs to be explored further. One possible explanation is that the positively charged DNR could assemble on the surface of the negatively charged PLA nanofibers, probably via the LBL mode and this may readily lead to the enhanced biomolecular interaction of the target drug molecules with some specific proteins or other related recognition factors that are embedded in the cellular membrane of the tumor cells, thus making the pathways of the drug delivery more efficient and finally facilitating the drug accumulation in cancer cells.

**Confocal Fluorescence Microscopy Studies of the Enhanced Cellular Uptake of DNR.** On the basis of the observations above, we further explored the potential application of the blending of Fe_3O_4 nanoparticles–PLA nanofibers. The Fe_3O_4 magnetic nanoparticles were imported in the system to improve the controllability in the survey of the drug delivery efficiency of anticancer agents.

As shown in Figure 3, after the leukemia K562 cancer cells were treated by DNR in the presence of Fe_3O_4 magnetic nanoparticles (Figures 3b and 3f), the number of living cancer cells apparently decreased, while the obvious green fluorescence was observed inside the cells. If the cells were treated in the presence of PLA nanofibers by the same means (Figures 3c and 3g), the intracellular fluorescence was even brighter. Since both the PLA nanofibers and the Fe_3O_4 nanoparticles gave no fluorescence, the observed green intracellular fluorescence was
only generated by the anticancer drug DNR. In contrast, when the target cells were treated with DNR alone (Figures 3a and 3e), the number of living cells did not decrease evidently and much weaker intracellular fluorescence could be observed than that in the presence of Fe₃O₄ nanoparticles, indicating the relatively lower DNR uptake efficiency to the target leukemia K562 cells without the participation of the nanostructures. Sequentially, much stronger intracellular fluorescence appeared in the target cells treated by DNR together with the Fe₃O₄-PLA nanocomposites (Figures 3d and 3h), which suggested that the nanocomposites could provide more efficient assistance in the drug delivery than the respective nanomaterials. Moreover, it was noted that when the individual leukemia K562 cell was treated by DNR together with the Fe₃O₄-PLA nanocomposites, the cancer cell shape changed obviously, implying the presence of the new nanocomposites could lead to the apparent swelling of most target leukemia cells.

The relative fluorescence intensity was given in Figure 4 to make a quantitative comparison of the four cases mentioned above. The intensity was obtained by sampling the fluorescence of each image and transforming the color value of each pixel to a gray value with our homemade program. The results illustrated that the intracellular fluorescence intensity in the presence of Fe₃O₄ nanoparticles, PLA nanofibers, and Fe₃O₄-PLA nanocomposites produced 88%, 216%, and 272% enhancement compared to the case of the cell treated with DNR alone, respectively. These intensity data indicated that the Fe₃O₄ or/and PLA nanofibers undoubtedly help the drugs enter into the cancer cells. Yet the mechanism, such as whether the nanomaterials were cytophaged in the cells, still needed to be explored.

**Electrochemical Characterization.** Additionally, to further explore the relative interaction mechanism, the differential pulse voltammetry (DPV) was utilized to investigate the interaction between DNR and leukemia K562 cells in the presence of various nanomaterials. Figure 5 illustrates the electrochemical study of DNR residue outside leukemia K562 cells in the presence of the Fe₃O₄ nanoparticles (Figure 5c), the PLA nanofibers (Figure 5d), and the Fe₃O₄-PLA nanocomposites (Figure 5e), respectively. The redox currents of the DNR residue outside leukemia K562 cells all decreased greatly from that in the absence of nanomaterials, with the following sequence of decrement: the Fe₃O₄-PLA nanocomposites > the PLA nanofibers > the Fe₃O₄ nanoparticles. Obviously, the decrement in the DPV currents is directly relative to the uptake amount in the cancer cells. These observations are consistent with the results of our fluorescence microscopy studies, indicating the remarkable synergistic effect of the novel Fe₃O₄-PLA nanocomposites in facilitating the accumulation of DNR. **Figure 3.** Typical images of the confocal fluorescence microscopy of drug-sensitive leukemia K562 cells treated with DNR (a, e); DNR and Fe₃O₄ nanoparticles (b, f); DNR and PLA nanofibers (c, g); DNR, Fe₃O₄-PLA nanocomposites (d, h). Parts (a)–(d) show the panoramic images of leukemia K562 cells. Parts (e)–(h) illustrate the typical individual single-cell images amplified from (a)–(d). The concentrations of DNR, Fe₃O₄ nanoparticles, and PLA are 7.5 × 10⁻⁵ mol/L, 2.3 × 10⁻⁴ mg/mL, and 2.5 × 10⁻⁴ mg/mL, respectively. Scale bar dimensions are provided for each image.

**Figure 4.** Comparison of the respective average intracellular fluorescence intensity of target leukemia K562 cells upon application of the DNR and respective nanomaterials. The excitation and emission fluorescence wavelength of the DNR is 480 and 560 nm, respectively.

**Figure 5.** Differential pulse voltammetric (DPV) detection of DNR (2.5 × 10⁻⁴ mol/L) alone (a) and DNR residue outside the drug-sensitive leukemia K562 cells without nanostructures (b) or in the presence of Fe₃O₄ nanoparticles (c) or PLA nanofibers (d) or Fe₃O₄-PLA nanocomposites (e). All concentrations of drugs and nanostructures are the same as those in Figure 3. The DPV parameters were set as follows: pulse amplitude, 0.05 V; pulse width, 0.05 s; pulse period, 0.1 s.
on drug-sensitive leukemia cells. These results demonstrated that the nanocomposites may act as the role of an effective loader to realize the inhibition of the growth of the respective leukemia cells even at a low drug concentration.

Cytotoxicity Research. The MTT assays were carried out for the cytotoxicity research. The inhibition rates for the cell system cultured with $9.93 \times 10^{-7}$ and $1.99 \times 10^{-6}$ mol/L DNR alone ($a$ and $a'$, Figure 6) were 16% and 28%, respectively. The cell inhibition with the same DNR concentrations in the presence of Fe$_3$O$_4$ nanoparticles ($b$ and $b'$, Figure 6) or PLA nanofibers ($c$ and $c'$, Figure 6) produced no significant difference from that of the cell treated with DNR alone. However, for DNR concentrations at $9.93 \times 10^{-7}$ and $1.99 \times 10^{-6}$ mol/L, the inhibition rates were 31% and 46% for the cell system cultured with DNR and Fe$_3$O$_4$−PLA ($d$ and $d'$, Figure 6), respectively. Therefore, both observations clearly showed the remarkable enhancement of the cell inhibition rates by Fe$_3$O$_4$−PLA nanocomposites, indicating the synergetic effect of the novel Fe$_3$O$_4$−PLA on the permeation and accumulation of DNR in the drug-sensitive leukemia cells.

Possible Mechanism of the Enhanced Effect by Nanocomposites. Based on the studies above, it appears that the self-assembly or attachment of the anticancer drug DNR to the nanocomposites can help to inhibit the drug-resistant effect of drug-cultured K562 cells and facilitate the uptake of anticancer drug DNR into the targeted tumor cells. Scheme 1 illustrates the possible mechanism of the enhanced effect on the drug uptake of DNR in target cancer cells by the Fe$_3$O$_4$−PLA nanocomposites. As is known, the PLA nanofibers have carboxyl groups on their surface; hence, the surface of the PLA nanofibers may be negatively charged at neutral pH solution. Then the positively charged DNR could be self-assembled onto the surface of PLA nanofibers by electrostatic interactions under certain pH conditions, leading to the enrichment of drug to a certain extent. On the other hand, nanoparticles could present a versatile nanoscale surface for biomolecular recognition due to the numerous potential benefits by merging biomacromolecules and nanoparticles. The tetraheptylammonium lipid shell of the Fe$_3$O$_4$ nanoparticles may help them inlay into the bilayer phospholipids membrane. Immediately, the electrostatic interaction between the positively charged Fe$_3$O$_4$ nanoparticles nailed on the membrane and the negatively charged surface of DNR-enriched PLA nanofibers may facilitate the accumulation of DNR on cellular membrane and thus lead to a remarkably increased diffusion of anticancer agents across the plasma membrane of the cancer cells. Through the synergetic effect of the membrane inset (Fe$_3$O$_4$) and the drug loader (PLA), the nanocomposites show a more excellent enhancing capability than that of the monocomponent nanomaterials. Our observations are in coincidence with this proposed mechanism.

Conclusions

In summary, the Fe$_3$O$_4$−PLA nanocomposites were prepared and adopted in the respective biorecognition and target drug delivery in this report. The potential application of the Fe$_3$O$_4$−PLA nanocomposites with widely used antileukemia drug, DNR,
in the treatment of the drug-sensitive leukemia K562 cells was explored. The MTT assay and optical, fluorescent, and electrochemical observations demonstrated that the novel nanocomposites could facilitate the interaction of anticancer drug DNR to targeted cancer cells with enormous enhancement in the relative biomolecular recognition and the accumulation of anticancer drug in the individual leukemia cell. Considering that the Fe₃O₄–PLA nanocomposites could remarkably enhance the diffusion efficiency of anticancer drugs into target tumor cells via the fix of Fe₃O₄ nanoparticles at the ailing area by external magnetic field during the tumor treatment, the chemotherapy effect could be effectively enhanced by combination of the application of the new nanocomposites in drug delivery systems for achieving targeted and controlled drug release. This may offer the possibility of the utilization of the nanocomposite to increase the local drug concentration of the target tumors and hence the efficiency of the cancer treatments. Thus, the results afford a new perspective for the targeted therapeutic approaches of cancers.

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**Supporting Information Available:** Size distribution histogram and TEM image of Fe₃O₄ nanoparticles, and excitation and emission spectra of DNR. This material is available free of charge via the Internet at http://pubs.acs.org.

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