Article

Synthesis of Europium-doped Fluorapatite Nanorods and Their Multifunctional Biomedical Applications

Hai Feng Zeng [1], Mu Yang Sun [2], Su Fan Wu [1,*] and Hai Feng Chen [2,*]

[1] Department of Plastic Surgery, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College; y-zhf@163.com

[2] Department of Biomedical Engineering, College of Engineering, Peking University; haifeng.chen@pku.edu.cn

* Correspondence: sufanwu@163.com (S.F.W.); haifeng.chen@pku.edu.cn (H.F.C.); Tel.: +86-0571-85893228 (S.F.W.); Tel.: +86-010-62754396 (H.F.C.).

Abstract: Europium (Eu)-doped fluorapatite (FA) nanorods have a biocompatibility similar to that of hydroxyapatite (HA) for use as cell imaging biomaterials due to their luminescent property. Here, we discuss the new application of europium-doped fluorapatite (Eu-FA) nanorods as an anticancer drug carrier. The Eu-FA nanorods were prepared by using a hydrothermal method. The morphology, crystal structure, fluorescence and composition were investigated. The specific crystal structure enables the effective loading of drug molecules. Doxorubicin (DOX), which was used as a model anticancer drug, effectively loaded onto the surface of the nanorods. The DOX release was pH dependent and occurred more rapidly at pH 5.5 than pH 7.4. The intracellular penetration of the DOX-loaded Eu-FA nanorods (Eu-FA/DOX) can be imaged in situ due to the self-fluorescence property. Treatment of melanoma A375 cells with Eu-FA/DOX elicited a more effective apoptosis rate than direct DOX treatment. Overall, Eu-FA exhibits potential for tracking and treating tumors and may be potentially useful as a multifunctional carrier system to effectively load and sustainably deliver drugs.

Keywords: lanthanides; fluorapatite; drug loading; nano carrier.

1. Introduction

Fluorapatite (FA) is one of the inorganic constituents of bone or teeth and is used for hard tissue repairs and replacements[1]. FA ($Ca_{10}(PO_4)_6F_2$, FA) can be synthetically prepared from hydroxyapatite and contains a suitable molecular concentration of OH- groups and F^- ions[2]. When doped with lanthanides (Eu^{3+} Tb^{3+} or Gd^{3+}), this material exhibits special photoluminescent and has the potential for application in biomedical studies[3-5]. Some lanthanide ion-doped materials (Eu^{3+} or Tb^{3+}) can emit visible fluorescence (i.e., green or red) when excited by ultraviolet or blue excitation wavelengths. In comparison to fluorescent dyes or semiconductor quantum dots (QDs), these lanthanide-doped nanoparticles are advantageous due to their photostability, sharp visible emission bandwidth, and nontoxicity[6-8]. In previous cell labeling and tracking studies, lanthanide ion-doped fluorapatite exhibited long excited state lifetimes and no toxicity to bone marrow mesenchymal stem cells[9,10].

Their high surface-to-volume ratio, reactivities, and biocompatibility make FA nanorods favorable for use in drug delivery studies[11,12]. In this study, Eu-FA nanorods were prepared and characterized. In addition, the obtained multifunctional Eu-FA nanorods were employed as a drug delivery carrier to investigate their drug loading and release properties using doxorubicin (DOX) as a model drug. Melanoma A375 cells were treated with DOX-loaded Eu-FA nanorods (Eu-FA/DOX) to confirm that Eu-FA/DOX induced apoptotic behavior in the tumor cells. The loading capacity of DOX and its release profile were investigated, and the in vitro biological efficacy of the intracellular delivery system was confirmed.

2. Results and discussion

The TEM results indicate that the Eu-FA crystals are on the nanoscale with a rod-like morphology (Fig. 1A). The TEM image also indicates that the Eu-FA nanorods are straight with a uniform morphology and good crystallinity. The diameters of the Eu-FA nanorods are 20-40 nm, with lengths ranging from 100 to 170 nm. Fig. 1B shows the nanoparticle powders under UV light in an Eppendorf tube and a converted fluorescence microscope. Fig. 1C shows the X-ray diffraction patterns (XRD) of the synthesized Eu-FA nanorods. All of the diffraction peaks can be indexed to the classical hexagonal phase according to ICDD 15-0876. The sharp characteristic peaks at approximately 25.91 and 31.91 correspond to the (002) and (211) lattice planes. Fig. 1D indicates that the emission intensity of Eu-FA is 591 ($^5D_0$-$^7F_1$) nm and 617 nm ($^5D_0$-$^7F_2$) upon excitation at 405 nm.

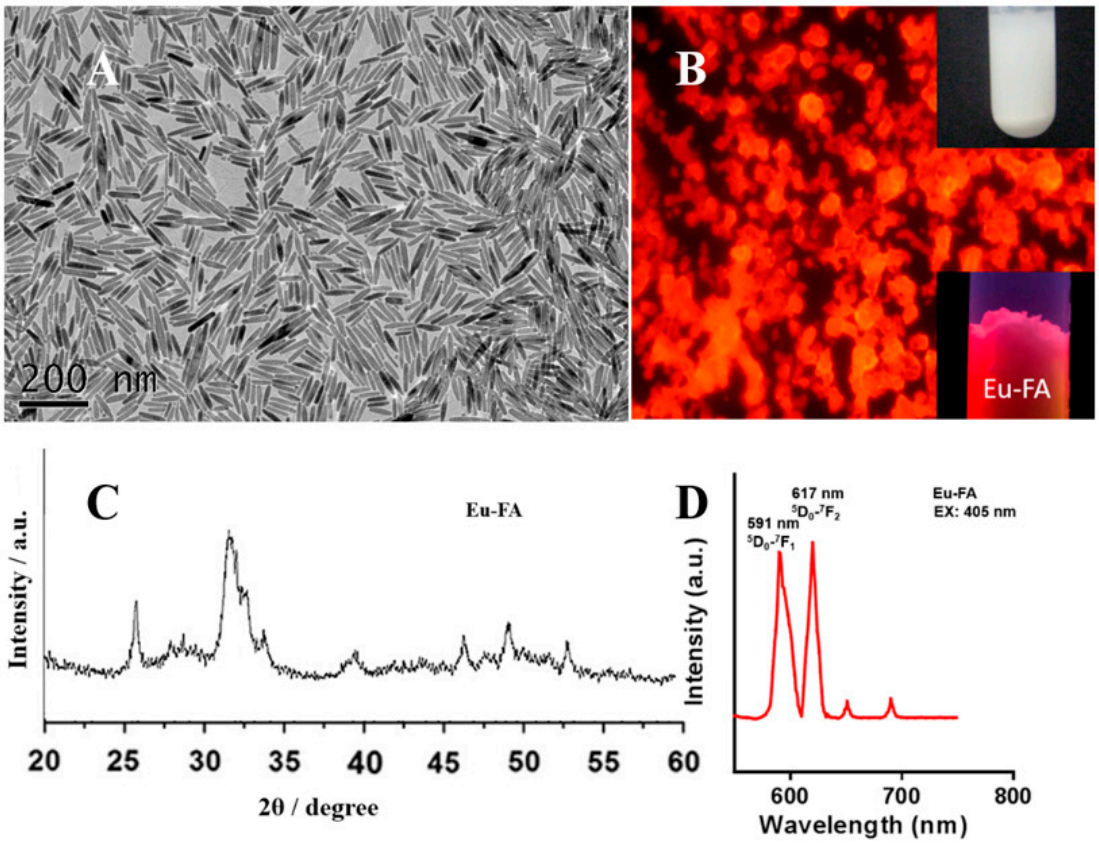

Figure 1. (A) TEM results for the Eu-FA crystals indicate a rod-like morphology; (B) Eu-FA nanorod powder under UV light and fluorescence microscopy; (C) XRD patterns correspond to a classical hexagonal phase; (D) Emission intensity of Eu-FA excited at 405 nm.

The DOX loading was recorded at a given nanocarrier content (2 mg) while varying the DOX concentrations from 0 to 5000 μg mL^{-1}. The DOX loading amount increased gradually as the DOX concentration increased, and a maximum loading of 43.7% was achieved (Fig. 2). The DOX molecules can adsorb onto the surface of the Eu-FA nanorods. The OH groups on the surface are most likely the reaction sites for the formation of hydrogen bonding with DOX as its adsorbed onto the surface[12]. DOX has a pKa of 8.3 and is positively charged at a pH of 7.4. Therefore, DOX is attracted to the negatively charged nanocarriers without additional surface functionalization[13].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 April 2017 doi:10.20944/preprints201704.0028.v1

Peer-reviewed version available at Molecules 2017, 22, , 753; doi:10.3390/molecules22050753

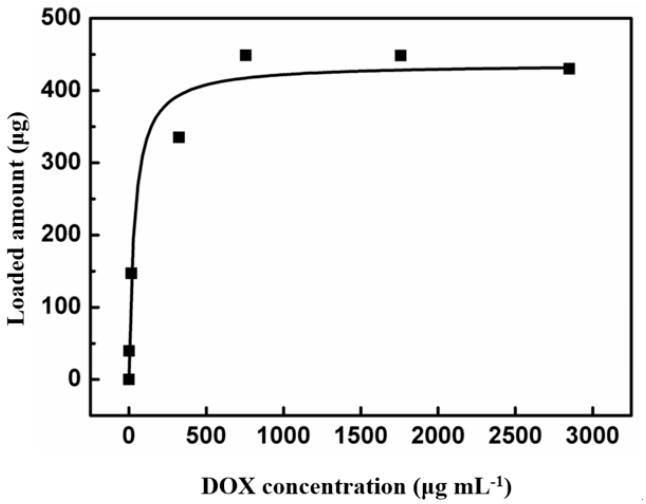

Figure 2. DOX loading curve for Eu-FA, which was obtained by measuring the amount of DOX loaded onto the nanorods.

The loaded DOX was subsequently released into PBS with different pH values. Although a pH of 7.4 is representative of normal physiological conditions, an acidic pH of 5.5 is similar to that of the extracellular tissues of tumor cells[14]. The DOX molecules exhibited a significant pH-dependent release (Fig. 3). This pH-sensitive phenomenon may be due to an increase in the hydrophilicity and solubility of DOX in acidic environments due to the stronger protonation of the -NH_2 groups in the DOX molecules[15]. Low drug release efficacy is favorable for drug delivery, and the high DOX release rate in acidic environments is beneficial for killing tumor cells. The results of the drug release tumor cell apoptosis test confirmed this viewpoint.

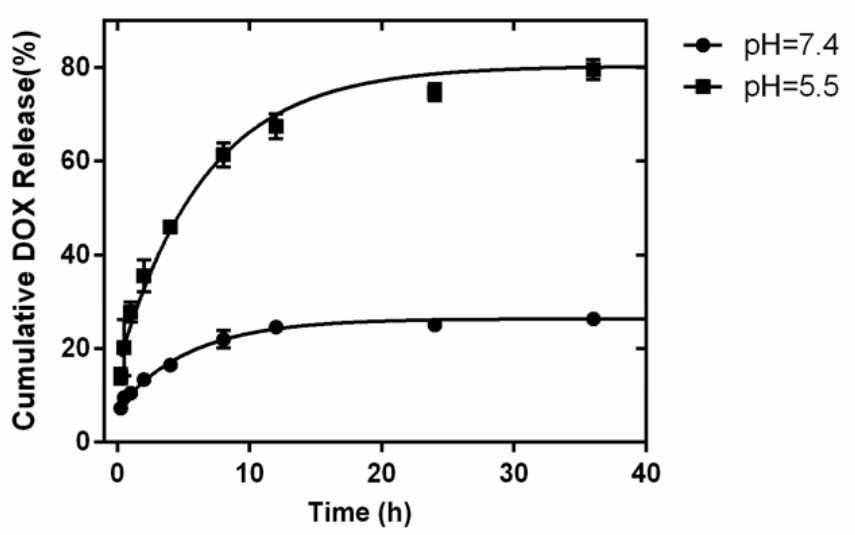

Figure 3. DOX release with different pH values (pH=5.5, 7.4).

Next, the cellular uptake of Eu-FA was examined. The A375 cells were treated with 10 μg of Eu-FA nanorods for 4 h. The intracellular penetration of Eu-FA/DOX can be imaged in situ due to the self-fluorescence property. The location of the Eu-FA nanorods in the A375 cells was confirmed by confocal laser scanning microscopy. The red regions represent the fluorescence signal of Eu-FA, and the blue signals arise from the DAPI-stained nucleus. The red fluorescence signals of Eu-FA were observed in the cytoplasm (around the blue signals) or within the nucleus (coincident with the blue signals) of the A375 cells treated with DOX (Fig. 4B). Therefore, only 4 h of treatment resulted in rapid uptake of the Eu-FA nanorods by the cells.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 April 2017 doi:10.20944/preprints201704.0028.v1

Peer-reviewed version available at Molecules 2017, 22, , 753; doi:10.3390/molecules22050753

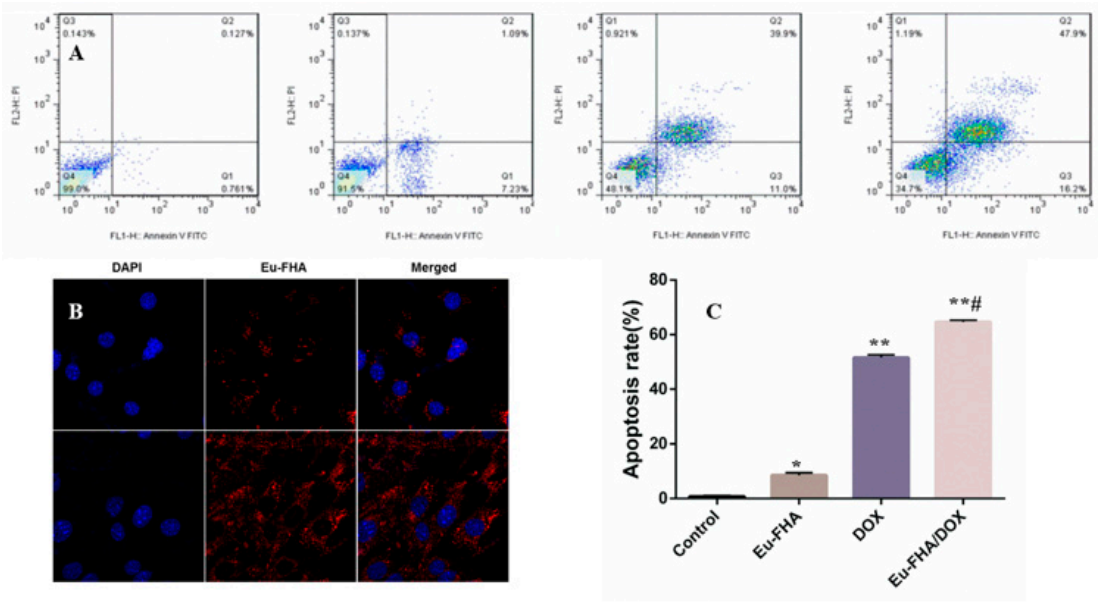

Figure 4. (A) Flow cytometry results for apoptosis after the A375 cells were treated with the control, Eu-FA, DOX and Eu-FA/DOX; (B) Blue fluorescence from DAPI and red fluorescence from Eu-FA were observed in the cytoplasm or within the nucleus; (C) Statistical results of apoptosis in each group (*# means $P < 0.05$, ** means $P < 0.01$).

To investigate the apoptosis rate induced by Eu-FA/DOX, the treated cells were double stained with FITC-Annexin V and PI followed by analysis with flow cytometry. Propidium iodide (PI) was used in conjunction with Annexin V to determine if the cells were viable, apoptotic, or necrotic from differences in the plasma membrane integrity and permeability. This approach is a universal and accurate method for detecting cell apoptosis[16]. As shown in (Fig 4A&4C), few apoptotic cells were detected in the untreated control. However, cell apoptosis was induced in the groups treated with DOX and Eu-FA/DOX (i.e., approximately 50.9% and 64.1%, respectively). Interestingly, more apoptosis cells were observed with the Eu-FA/DOX treatment group compared to that with the DOX-only treatment group, and this difference was statistically significant. This means more apoptosis was observed when DOX was uploaded by the Eu-FA nanocarrier system. Therefore, in the group treated with Eu-FA, 8.31% of the population exhibited apoptotic cells. The non-apoptotic cell fraction was considered to be necrotic (i.e., dying from a cause other than apoptosis). At this point, the cell death mechanism of the A375 cells treated with DOX is considered to be different from that of cells treated with Eu-FA/DOX.

3. Materials and Methods

3.1 Synthesis of Eu-FHA nanorods

Eu^{3+} doped FHA nanoparticles were synthesized via a hydrothermal method (Synthesized by the Institute of Biomedical Engineering, Peking University). All chemicals were analytical grade. 0.5 g of octadecylamine were dissolved in 4 mL of oleic acid with heating, and then, 16 mL of ethanol and an aqueous solution of $Ca(NO_3)_2$ (0.28 M, 7.0 mL) were added with stirring. NaF (0.24 M, 2.0 mL), $Eu(NO_3)_3$ (0.20 M, 2.0 mL) and Na_3PO_4 (0.20 M, 7.0 mL) were added to the solution followed by an additional 5 min of stirring. Then, the sealed flask was heated at 160 °C for 16 h. The obtained mixture consisted of FA nanoparticles doped with Eu^{3+}, and these nanoparticles were collected by centrifugation and freeze-dried.

3.2 Characterization of Eu-FHA nanorods

Transmission electron microscopy (TEM) was carried out on a JEM-2100 instrument to determine the sizes and morphologies of the Eu-FHA nanorods. The samples were prepared by placing a drop of a dilute ethanol dispersion of the products on the surface of a copper grid. XRD analysis was performed using an Image Plate X-ray Diffractometer (RAPID-S, Rigaku, Japan) with Cu Ka radiation. The luminescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. Luminescent photography of the sample was performed under UV light at 390 nm.

3.3 Preparation of the drug loading and release system

The drug loading and release system using Eu-FA nanorods as the carrier was prepared using an absorption method. 2 mg of the Eu-FA sample were added to 400 μL of a PBS solution at room temperature. Doxorubicin hydrochloride (DOX, Shanghai Sangon) was dissolved in the PBS solution at a pH of 7.4 to produce stock solutions with concentrations of 0, 200, 750, 2000, 3000, 4000, and 5000 μg mL^{-1}. A standard curve was obtained using a UV-Vis spectrophotometer with an absorbance of 483 nm to quantify the drug loading amount.

To study the release curve of DOX from the nanocarrier samples, the as-prepared Eu-FA/DOX nanorods were used. To test the DOX release kinetics, 2 mg of each sample were dispersed in 2 mL of PBS prepared with different pH values (5.5 and 7.4) followed by incubation at 37 °C. At different time points, the amount of drug released into the medium was measured.

3.4 Cell culture and fluorescence imaging

Melanoma A375 cells were seeded at 10^5 cells in each well of 6-well culture plates and treated with Eu-FA nanorods for 4 h. The fluorescent images were observed and analyzed using a laser scanning confocal microscope (LSM780, Leica). The cells were counterstained with DAPI (Invitrogen, USA) to observe the nucleus.

3.5 Cell apoptosis detection

The A375 cells treated with Eu-FA, DOX or Eu-FA/DOX for 24 h were harvested. Flow cytometry was employed to detect cell death and apoptosis according to the manufacturer's instruction. After being harvested via trypsinization and lysed with ice-cold PBS, the cocultured cells with Annexin V-FITC (50 μg/mL, Sigma, USA) were incubated for 15 min in a dark at room temperature. Then, the re-suspended cells were stained with propidium iodide (10 μg/mL, Sigma, USA). A Becton Dickinson FACS/Calibur cytometer (Becton Dickinson Biosciences) was employed to analyze the Annexin V-FITC and propidium iodide signals.

3.6 Statistics analysis

Statistical analysis was performed using the SPSS 22.0 software. Student's t-tests were performed to determine the statistical significance between experimental groups. $P < 0.05$ was considered to indicate statistical significance. All results are expressed as the mean ± standard.

4. Conclusions

In summary, the as-synthesized Eu-FA nanorods exhibited excellent photoluminescence and high drug loading capacity. We believe that this multifunctional nanocarrier will generate further interest and open new avenues for research on the applicability of using these nanoparticles in cancer treatment.

Acknowledgments: The project was supported by zhejiang provincial natural science foundation for young Scientists (Q16C100002).

Author Contributions: Hai Feng Zeng and Hai Feng Chen conceived and designed the experiments; Hai Feng Zeng performed the experiments; Su Fan Wu analyzed the data; Mu Yang Sun contributed materials; Hai Feng Zeng wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wu, Y.J.; Tseng, Y.H.; Chan, J.C.C. Morphology control of fluorapatite crystallites by citrate ions. *Crystal Growth & Design* **2010**, *10*, 4240-4240.
2. Jantová, S.; Theiszová, M.; Letasiová, S.; Birosová, L.; Palou, T.M. In vitro effects of fluor-hydroxyapatite, fluorapatite and hydroxyapatite on colony formation, DNA damage and mutagenicity. *Mutation Research/fundamental & Molecular Mechanisms of Mutagenesis* **2008**, *652*, 139-144.
3. Chen, F.; Huang, P.; Zhu, Y.J.; Wu, J.; Zhang, C.L.; Cui, D.X. The photoluminescence, drug delivery and imaging properties of multifunctional eu3+/gd3+ dual-doped hydroxyapatite nanorods. *Biomaterials* **2011**, *32*, 9031-9039.
4. Wang, F.; Liu, X. Multicolor tuning of lanthanide-doped nanoparticles by single wavelength excitation. *Accounts of chemical research* **2014**, *47*, 1378-1385.
5. Liu, Y.; Tu, D.; Zhu, H.; Chen, X. Cheminform abstract: Lanthanide-doped luminescent nanoprobes: Controlled synthesis, optical spectroscopy, and bioapplications. *Chemical Society Reviews* **2013**, *42*, 6924.
6. Feng, R.; Jiang, F.L.; Wu, M.Y.; Chen, L.; Yan, C.F.; Hong, M.C. Structures and photoluminescent properties of the lanthanide coordination complexes with hydroxyquinoline carboxylate ligands. *Crystal Growth & Design* **2010**, *10*, 2306-2313.
7. Ou, Y.C.; Gao, X.; Zhou, Y.; Chen, Y.C.; Wang, L.F.; Wu, J.Z.; Tong, M.L. Magnetic properties and photoluminescence of lanthanide coordination polymers constructed with conformation-flexible cyclohexane-tetracarboxylate ligands. *Crystal Growth & Design* **2015**, *16*.
8. Kariem, M.; Yawer, M.; Sharma, S.; Sheikh, H.N. Syntheses, crystal structure, luminescence, porosity and magnetic properties of three-dimensional lanthanide coordination polymers with 2-aminoterepthalic acid. **2016**, *1*, 4489-4501.
9. Zeng, H.; Li, X.; Xie, F.; Teng, L.; Chen, H. Dextran-coated fluorapatite nanorods doped with lanthanides in labelling and directing osteogenic differentiation of bone marrow mesenchymal stem cells. *Journal of Materials Chemistry B* **2014**, *2*, 3609-3617.
10. Li, X.; Zeng, H.; Teng, L.; Chen, H. Comparative investigation on the crystal structure and cell behavior of rare-earth doped fluorescent apatite nanocrystals. *Materials Letters* **2014**, *125*, 78-81.
11. Rulis, P.; Ouyang, L.; Ching, W.Y. Electronic structure and bonding in calcium apatite crystals: Hydroxyapatite, fluorapatite, chlorapatite, and bromapatite. *Physical Review B* **2004**, *70*, 2806-2810.
12. Saber-Samandari, S.; Nezafati, N.; Saber-Samandari, S. The effective role of hydroxyapatite based composites in anticancer drug delivery systems. *Critical Reviews in Therapeutic Drug Carrier Systems* **2016**, *33*, 41.
13. Sanson, C.; Schatz, C.; Meins, J.F.L.; Soum, A.; Thévenot, J.; Garanger, E.; Lecommandoux, S. A simple method to achieve high doxorubicin loading in biodegradable polymersomes. *Journal of Controlled Release* **2010**, *147*, 428-435.
14. Lee, E.S.; Gao, Z.; Bae, Y.H. Recent progress in tumor ph targeting nanotechnology. *Journal of Controlled Release Official Journal of the Controlled Release Society* **2011**, *132*, 164-170.
15. Li, H.; Bian, S.; Huang, Y.; Liang, J.; Fan, Y.; Zhang, X. High drug loading ph-sensitive pullulan-dox conjugate nanoparticles for hepatic targeting. *Journal of Biomedical Materials Research Part A* **2014**, *102*, 150.
16. Rieger, A.M.; Nelson, K.L.; Konowalchuk, J.D.; Barreda, D.R. Modified annexin v/propidium iodide apoptosis assay for accurate assessment of cell death. *Journal of Visualized Experiments* **2011**, *50*, 40-40.