Research Note

A Novel Biodegradable Hollow Nanocarrier Consisting Superparamagnetic \( \text{Fe}_3\text{O}_4 \)-loaded poly-\( \gamma \)-glutamic Acid and Chitosan Oligosaccharide for Targeted Delivery of Sulforaphane from Broccoli Seed Extracts

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( Accepted for publication, May 31, 2019)

Abstract: The aim of this study was to synthesize a polymeric nanocarrier system loaded with superparamagnetic iron oxide nanoparticles (SPION) and the sulforaphane (SFN) through a cross-linking method in order to protect the SFN. According to this experiment, the drug-loading content and encapsulation efficiency were 29.97±0.08% and 0.34±0.41%, respectively. The total cumulative amount of SFN, released from the nanocarrier in vitro was 87.90% over a 72 h period. MTT assays indicated that the SPION-SFN-loaded nanocarrier exhibited a much higher in vitro antitumor efficacy than free SFN. Together, these results indicate that SPION-loaded nanocarrier exhibits good drug-loading ability and sustained-release function.

Key words: Superparamagnetic iron oxide nanoparticles, Poly-\( \gamma \)-glutamic acid, Chitosan oligosaccharide, Nanocarrier, Sulforaphane

Introduction

Here we report a novel biodegradable hollow nanocarrier consisting of two types of weak polyelectrolytes: chitosan oligosaccharide (CO), a cationic polyelectrolyte, and poly-\( \gamma \)-glutamicacid (\( \gamma \)-PGA), an anionic polyelectrolyte. \( \gamma \)-PGA is composed of a racemic mixture of naturally occurring D- and L- glutamic acids \( \gamma \)-linked through amide bonds, and attracts particular attention as one of the most promising biomaterials for medical use. \( \gamma \)-PGA is a hydrophilic polypeptide that can be used as a carrier of various drugs and biomolecules, such as proteins, growth factors, and oligonucleotides, and its degradation product is in fact nutritional to cellular proliferation. Based on the positively charged surface of CO and the negatively charged one of \( \gamma \)-PGA, CO/\( \gamma \)-PGA polyelectrolyte complexes is benefited to us to form various kinds of nanomaterials. CO and \( \gamma \)-PGA composites, such as nanoparticles, hydrogels, and microcapsules, have been reported as drug carriers. CO are biocompatible and biodegradable, and demonstrate antibacterial effects. CO and its derivatives are promising candidates for drug carriers and have been applied in many biomedical fields.

Superparamagnetic iron oxide nanoparticles (SPIONs) have also been extensively investigated for biomedical applications because of their advantages, including easy synthesis, small size, low toxicity, and unique magnetic properties, and have been approved by US Food and Drug Administration (FDA) for disease imaging. In recent years, several studies have demonstrated the potential of SPION-mediated targeted delivery of various anticancer drugs. Superparamagnetic nanoparticles may be incorporated within drug carrier systems to facilitate the manipulation and delivery of drug-loaded nanocarriers to a desired area through externally localized magnetic steering. James Wainaina et al. reported the synthesis of composite nanoparticles of magnetite and an amphiphilic polymer that can be used for cancer diagnosis and therapy. Shveta Mahajan et al. developed folate receptor-targeted SPI-ON-polymer micelle hybrids that could be used as therapeutic contrast enhancers for imaging folate-receptor overexpressing cancers.

Sulforaphane (1-isothiocyanate-4R-(methylsulfinyl) butan, SFN) is an isothiocyanate derivative, of cruciferous vegetables, resulting from the hydrolysis of its precursor glucoraphanin (4-methylsulfinylbutyl glucosinolate) by the enzyme myrosinase. Sulforaphane interest has increased in the pharmaceutical and nutraceutical industries due to its anticancer and chemopreventive effects, but is very sensitive to changes in temperature and pH.

In the present investigation, a polymeric nanocarrier was fabricated by simultaneously encapsulating magnetite nanoparticles and SFN into the core of nanocarriers composed of \( \gamma \)-PGA and CO. The physical properties of the nanoparticles, including their size, morphology, and in vitro drug release behavior were comprehensively characterized. SPI-ON-SFN-loaded exhibited a much higher in vitro antitumor efficacy than free SFN. These promising results indicate that targeted drug delivery could be achieved with nanocarriers under magnetic guidance.

Materials and methods

\( \gamma \)-PGA (40KD) was purchased from Sigma-Aldrich (St. Louis, MO). CO (degree of deacetylation 88%, 800-1,000KD) was purchased from Sigma-Aldrich. \( \text{Fe}_3\text{O}_4 \) magnetic nanoparticles with size of about 10 nm were purchased from Anhui Maanshan Powder Engineering Co. (Anhui, China). Broccoli was purchased from Shandong province, China. A549 cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences and cultured at the Life Science Institute of Qufu Normal University. All other chemicals were of analytical grade and were used as received without further purification.

Ultrasonic cell disruptor was purchased from Xinzhong Biological...
Company (Ningbo, China). Rotary evaporator was purchased from Yarong Biochemical Company (Shanghai, China). HPLC was purchased from Agilent (USA). Nicolet 5700 spectrometer was purchased from Thermo Electron Corporation (USA). Transmission Electron Microscope was purchased from Japanese electronics company (Japan). Mini Flex600 X-ray diffractometer was purchased from Rigaku company (Japan). SynergyH1 full-featured microplate inspection system was purchased from Berten Instruments (USA). Digital fluorescent inverted microscope was purchased from OLYMPUS company (Philippines).

Preparation of hollow nanocarrier
Hollow nanocarriers were prepared through a cross-linking method\cite{21}. γ-PGA (125 mg), CO (40 mg) and Fe₃O₄ nanoparticles (4 mg) were added to 10 ml of high-purity water at room temperature. The mixture was sonicated vigorously in a glass beaker and then stirred for 3 hours. To remove aggregated magnetite particles, the nanocarrier solution was filtered through a filter with a pore size of 1 μm. The resultant nanocarrier solution was stored at 4°C prior to analysis.

Extraction of sulforaphane
Hydrolysis of glucoraphanin was performed according to the method proposed by Campas-Baypoli et al. (2010)\cite{22}, with modifications. After the broccoli seeds were homogenized, 5 g of the broccoli seeds were weighed in 50 ml of ultrapure water, then sonicated for 30 minutes and placed in a 45°C water bath for 12 hours. Ethyl acetate (100 ml) was added accurately to the silica gel column to collect fractions.

Quantification of sulforaphane
Standard stock solutions of SFN were prepared by dissolving the substances in acetonitrile to a final concentration of 1 mg/ml. The standard working solutions were made by diluting the stock solutions with acetonitrile. The samples were filtered through 0.22 μm nylon membrane. A 0.02 ml aliquot of the mixture was injected into the HPLC system (Agilent 1260, Agilent Technologies, USA) for analysis of the SFN content. The isocratic mobile phase was acetonitrile: water (13:7 v/v), at a flow rate of 1.0 ml/min. The eluates were monitored at 254 nm\cite{23}. Each sample was eluted for a total of 3 min.

Preparation of SPION-SFN-loaded nanocarrier
SPION-SFN-loaded nanocarriers were prepared through a cross-linking method. SFN extracted from broccoli. SFN (50 mg) was dissolved in 10 ml DMSO, γ-PGA (125 mg), CO (40 mg) and Fe₃O₄ nanoparticles (4 mg) were added to 10 ml of high-purity water, then the two solutions were mixed at room temperature. The mixture was sonicated vigorously in a glass beaker and then stirred for 3 hours. To remove free sulforaphane and aggregated magnetite particles, the nanocarrier solution was filtered through a filter with a pore size of 1 μm. The resultant nanocarrier solution was stored at 4°C prior to analysis.

Characterization of the hollow and SPION-SFN-loaded nanocarrier
Fourier transform infrared spectral studies were performed on a Nicolet 5700 spectrometer equipped with an infrared microscope. Spectra were acquired between 4,000 and 500 cm⁻¹ with a resolution of 2 cm⁻¹. All the freeze-dried powder samples were tiled on the microstat for FTIR measurements. TEM analysis was performed on a JEM-2100PLUS at an operating voltage of 200 kV. TEM samples were prepared as follows: 2 μl of sample solution was placed on a carbon-coated 300 mesh copper grid and air-dried. The main test parameters of the X-ray diffractometer were: Cu target Kα radiation with a wavelength \( λ = 1.5418 \text{Å} \). A MiniFlex 600 X-ray diffraction instrument (XRD) was used for wide-angle scanning from 10° to 80°. XRD samples were prepared as follows: 10 ml of sample solution was freeze-dried and ground. Then a small amount of powder was taken for testing.

In vitro release of drugs from the SPION-SFN-loaded nanocarrier
The SFN release study was performed in an air bath shaker at 37°C\cite{24}. The solution of SPION-SFN-loaded nanocarrier (10 ml) was transferred to a dialysis bag (molecular weight cut-off: 8,000-10,000 Da, Spectra/Por, USA). The bags were placed respectively in 200 ml of PBS (pH=5.5). At selected time intervals, samples were removed from the solution inside the dialysis bag, dissolved in DMSO, centrifuged at 10,000 rpm for 30 min to remove the magnetite nanoparticles and subjected to HPLC with detection at 254 nm to determine the residual drug content. The amount of drug released was calculated from these measurements.

In vitro cytotoxicity study
Cancer cell lines (A549) was seeded in 96-well plates for 24 h before the MTT assay. Then, the medium was removed and was replaced by free SFN or by SPION-SFN-loaded nanocarrier at different concentrations, and the samples were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂. The result was analyzed on a microplate reader at 570 nm, and the IC₅₀ values (the concentration at which 50% of cells are killed) was calculated.

SPION-SFN-loaded nanocarrier were dissolved in DMSO and to concentrations of 40 μg/ml. Subsequently, the cell migration and invasion experiments were performed with DMSO as the control (three parallel experiments were performed for each group). Cell invasion and migration assays were conducted as previously described using the Transwell method\cite{25}.

Statistical analysis
All experiments were performed in triplicate and the data are reported as the mean values with standard deviations. Experimental data was statistically analyzed using SPSS statistics 20.0 statistical software. The differences between the control and the test groups were assessed with Student’s t-test. Differences were to be considered statistically significant when P<0.05.

Results
Effects of proportions of γ-PGA and CO on diameter and drug loading content of the S-PION-SFN-loaded nanocarrier
The nanocarrier were prepared using different proportions of γ-PGA and CO(m:m). As shown in Table 1, the drug loading content and the particle diameter were closely correlated with the proportions of γ-PGA and CO. The higher the content of CO, the diameter of the particles is larger, but the drug loading is hardly unchanged. Therefore, the proportion of 25:8 was chosen for further study.

Characterization of the hollow and SPION-SFN-loaded nanocarrier
Fig. 1 shows TEM images of the hollow nanocarrier (A) and SPION-SFN-loaded nanocarrier (B). A cluster of magnetite nanoparticles with a typical spherical morphology and an average size of 43 nm was
observed. This observation demonstrates that the clusters of magnetite nanoparticles were successfully incorporated into almost all of the polymeric nanocarriers.

Fig. 2 shows the FTIR spectra of the free SFN (A) and SPION-SFN-loaded nanocarrier (B). In the spectrum of the free SFN, the peak at 2,381 cm\(^{-1}\), belonging to telescopic absorption of \(-N=C=S\), the peak at 1,430 cm\(^{-1}\) is bending vibration of C=O, the peak at 1,116 cm\(^{-1}\) is the stretching vibrations on glucosides of C-O, the peak at 986 cm\(^{-1}\) is a stretching vibration of S=O and the peak at 828 cm\(^{-1}\) is a stretching vibration of C-S. The map shows that the extracted substance is SFN. The characteristic absorption bands of the free SFN were appeared in the spectrum of the SPION-SFN-loaded nanocarrier, SPION-SFN-loaded nanocarrier shows that the absorption peak at 2,372 cm\(^{-1}\), which was attributed to the decline on the \(-N=C=S\) of SFN. Bands characteristic of an amide in the region 1,576 cm\(^{-1}\) showing the interaction of the polymers, it is due to the amino groups of \(\gamma\)-PGA with the carboxyl groups of the CO. Both spectra show the band belonging to the C-O group on glucoside vibration in the region 1,030 cm\(^{-1}\) characteristic of SFN, confirming the presence of the SFN in both complexes.

Fig. 3 shows the XRD of the Fe\(_3\)O\(_4\) and hollow nanocarrier. In the map of the Fe\(_3\)O\(_4\), characteristic peaks at 35.54°, 57.1°, 62.7° also appeared in the hollow nanocarrier, however, the intensity of these characteristic peaks were decreased. This may be due to the CO/\(\gamma\)-PGA coated on the Fe\(_3\)O\(_4\) surface.
**In vitro drug release behavior**

The release behavior of the SPION-SFN-loaded nanocarrier was investigated in PBS (pH=6.81). As shown in Fig. 4, an initial burst of released of drugs was observed in the first 8 h, followed by a sustained and slow release over the next more than a dozens hours. SPION-SFN-loaded nanocarrier released slowly over a 72 h period, compared to free drugs, which continued to decline after a maximum release rate of the first few hours, with a close to zero release rate at 32 h. The initial burst of released of drugs from the nanocarrier may be attributed to drugs molecules adsorbed on to the surface of the nanocarrier and located at the interface between the hydrophobic core and the inner hydrophilic layer. The subsequent sustained release rate of the drug is most likely because of the slow diffusion from the hydrophobic core and the gradual degradation of the copolymer. The total cumulative amount of SFN released from the nanocarrier was 87.90% over a 72 h period.

**In vitro cytotoxicity study**

The in vitro antitumor effects of the hollow nanocarrier, free SFN and the SPION-SFN-loaded nanocarrier were evaluated in A549 cells with the MTT assay. As shown in Fig. 5, the SPION-SFN-loaded nano-
carrier showed a cytotoxicity slightly lower than that of free SFN. The IC_{50} (the concentration at which 50% of cells are killed) values calculated from the cytotoxicity data for the SPION-SFN-loaded nanocarrier and the free SFN were 30.80±0.01 and 17.30±0.47 μg/ml, respectively. The cell viability of the groups of SPION-SFN-loaded nanocarrier and the free SFN was significantly reduced compared to the control group (P<0.05). The lower IC_{50} value of the free SFN indicates that the free SFN rapidly diffuses into the cells and exerts antitumor activity whereas the SPION-SFN-loaded nanocarrier exhibits a more sustained and controlled SFN release in the intracellular compartments after cellular internalization. Interestingly, the hollow nanocarrier did not significantly affect tumor cell growth under the experimental conditions studied (up to 200 μg/ml). This result suggests that the hollow nanocarrier are not inherently cytotoxic and that the cytotoxicity was caused by the SFN. The ability to modify these nanoparticles using well-established chemistries elicits further control on the localization, biocompatibility and efficacy of these actively targeted systems in vivo.

Next, transwell assays were performed to investigate whether migration and invasion of A549 cells would be attenuated by SPI-ON-SFN-loaded nanocarrier. The results indicated that the number of cells in the transwell chamber of the SPION-SFN-loaded nanocarrier group was significantly reduced compared with the control group, indicating a significant decrease in the migration and invasion ability of A549 cells (P<0.05; Fig 6 and Fig 7).

**Discussion**

Based on the results of characterization of SPION-SFN-loaded nanocarriers, we can know that SFN and magnetite nanoparticles can be successfully encapsulated into the core of CO/γ-PGA copolymer nanocarriers by cross-linking method, and have cytotoxicity. Biopolymers, such as γ-PGA and CO, are safe and suitable materials for coating SFN extracts by cross-linking methods. It is commendable that the nanoparticles have a sustained release effect, and the patient can reduce the number of medications and reduce the pain. In addition, the superparamagnetic Fe₃O₄ core can further enhance the targeting of nanocarriers in vitro. Targeting can bring the drug directly to the lesion and avoid damage to normal cell tissue, thus bringing hope for effective treatment of cancer. Magnetically targeted nanoparticle carriers can direct drug delivery to a targeting site under the application of an exogenous magnetic field that will facilitate further development and evaluation of nanomedicine for targeted drug delivery and enhanced therapeutic efficacy.

**Acknowledgements**

The authors thank the grant from Natural Science Foundation of Shandong Province (ZR2014CM020). At the same time, thanks are due to Zhiang Liu for guidance drawing figures.
Conflict of Interest

Fund Project: Natural Science Foundation of Shandong Province (Project Label: ZR2014CM020).

References

1. Liang W, Yu A, Wang G, Zheng F, Jia J and Xu H. Chitosan-based nanoparticles of avermectin to control pine wood nematodes. Int J Biol Macromol 112: 258-263, 2018
2. Lu KY, Lin CW, Hsu CH, HoYC, Chuang EY, Sung HW and Mi FL. FRET-based dual-emission and pH-responsive nanocarriers for enhanced delivery of protein across intestinal epithelial cell barrier. J ACS Appl Mater Interfaces 6(20): 18275-18289, 2014
3. Liao ZX, Peng SF, Chiu YL, Hsiao CW, Liu HY, Lim WH, Lu HM and Sung HW. Enhancement of efficiency of chitosan-based complexes for gene transfection with poly(γ-glutamic acid) by augmenting their cellular uptake and intracellular unpackage. J Control Release 193: 304-315, 2016
4. Su YR, Yu SH, Chou AC, Wu JY, Lin YF, Lu KY and Mi FL. Preparation and properties of pH-responsive, self-assembled colloidal nanoparticles from guanidine-containing poly-peptide and chitosan for antibiotic delivery. Colloids Surf A Physicochem Eng Asp 494: 9-20, 2016
5. Teixeira GQ, Pereira CL, Castro F, Ferreira JR, Gomez-Lazaro M, Aguiar P, BarbosaMA, Neidlinger-Wilke C and Goncalves RM. Anti-inflammatory Chitosan/Poly-γ-glutamic acid nanoparticles control inflammation while remodeling extracellular matrix in degenerated intervertebral disc. Acta Biomater. 42: 168-179, 2016
6. Kim ES, Lee J and Lee HG. Nanoencapsulation of red ginseng extracts Using chitosan with poly glutamic acid or fucoidan for improving anthrithobic activities. J Agric Food Chem 64(23): 4765-4771, 2016
7. Park BG, Kang H, Lee W, Kim JS and Son T. Reinforcement of pH-responsive γ-poly (glutamic acid)/chitosan hydrogel for orally administrable colon-targeted drug delivery. J Appl Polym Sci 127(1): 832-836, 2013
8. Yan S, Rao S, Zhu J, Wang Z, Zhang Y, Duan Y, Chen X and Yin J. Nanoporous multi-aiyperpoly (L-glutamic acid)/chitosan microcapsules for drug delivery. Int J Pharm 427(2): 443-451, 2012
9. Thakur VK and Thakur MK. Recent advances in graft copolymerization and applications of chitosan: a review. ACS Sustainable Chem Eng 2(12): 2637-2652, 2014
10. Shi JJ, Xiao ZY, Kamaly N and Farokhzad OC. Self-assembled targeted nanoparticles: evolution of technologies and bench to bedside translation. Acc Chem Res 44(10): 1123, 2011
11. Kumar A, Jena PK, Behera S, Lockey RF, Mohapatra S and Mohapatra S. Multifunctional magnetic nanoparticles for targeted delivery. Nanomedicine: NBM 6(1): 64, 2010
12. Parmar KR, Patel I, Basha S and Murthy Z.V. Synthesis of acetone reduced graphene-oxide/Fe₃O₄ composite through simple and efficient chemical reduction of exfoliated graphene oxide for removal of dye from aqueous solution. J MATER SCI 49(19): 6772-6783, 2014
13. Bharali DJ and Mousa SA. Emerging nanomedicines for early cancer detection and improv-ed treatment: Current perspective and future promise. J Pharmacol Ther 128(2): 324, 2010
14. Luo B, Xu S, Luo A, Wang WR, Wang SL, Guo J, Lin Y, Zhao DY and Wang CC. Mesoporous biocompatible and acid-degradable magnetic colloidal nanocrystal clusters with sustainable stability and high hydrophobic drug loading capacity. J ACS Nano 5(2): 1428, 2011
15. Hua MY, Yang HW, Liu HL, Tsai RY, Pang ST, Chuang KL, Chang YS, Hung TL, Chang YH, Chuang HC and Chuang CK. Super-high-magnetization nanocarrier as doxorubicin delivery platform for magnetic targeting therapy. Biomaterials 32(34): 8999-9010, 2011
16. Chomoucka J, Drbohlavova J, Huska D, Adam V, Kizek R and Hulak E. Magnetic nanoparticles and targeted drug delivering. Pharmacol Res 62(2): 144-149, 2010
17. Ding G, Guo Y, Lv Y, Liu X, Xu L and Zhang X. A double-targeted magnetic nanocarrier with potential application in hydrophobic drug delivery. Colloids Surf B Biointerfaces 91: 68-76, 2012
18. Mahajan S, Koul V, Choudhary V, Shishodia G and Bharti AC. Preparation and in vitro evaluation of folate-receptor-targeted SPION-polymer micelle hybrids for MRI contrastenhancement in cancer imaging. Nanotechnology 24(1): 015603, 2013
19. García-Saldaña JS, Campas-Baypoli ON, López-Cervantes J, Sánchez-Machado DI, Cantú-Soto EU and Rodríguez-Ramírez R. Microencapsulation of sulforaphane from broccoli seed extracts by gelatin/gum arabic and gelatin/pectin complexes. J Food Chem 201: 94-100, 2016
20. Gupta KC and Jabrail FH. Glutaraldehyde cross-linked chitosan microspheres for controlled release of centchroman. Carbohydr Hydr Res 342(15): 2244-2252, 2007
21. Campas-Baypoli ON, Sánchez-Machado DI, Bueno-Solano C, Ramírez-Wong B and López-Cervantes J. HPLC method validation for measurement of sulforaphane levelin broccoli-products. Biomed Chromatogr 24(4): 387-392, 2010
22. Matushesski NV, Wallig MA, Juvik JA, Klein BP, Kushad MM and Jeffery EF. Preparative HPLC Method for the Purification of Sulforaphane and Sulforaphane Nitrile from Brassica oleracea. J Agric Food Chem 49(4): 1867-1872, 2001
23. Xiong XB, Mahmoud A, Uludag H and Lavasanifar A. Multifunctional polymeric micelles for enhanced intracellular delivery of doxorubicin to metastatic cancer cells. Pharm Res 25(11): 2555-2566, 2008
24. Kondo S, Iwata S, Yamada T, Inoue Y, Ichihara H, Kichikawa Y, Katayose T, Souta-Kuribara A, Yamazaki H, Hosono O, Kawasaki H, Tanaka H, Hayashi Y, Sakamoto M, Kamiya K, Dang NH and Morimoto C. Impact of the Integrin Signaling Adaptor Protein NEDD9 on Prognosis and Metastatic Behavior of Human Lung Cancer. Clin Cancer Res 18(2-2): 6326-6338, 2012.