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Research Article

Synthesis of ginsenoside Rb_1-imprinted magnetic polymer nanoparticles for the extraction and cellular delivery of therapeutic ginsenosides

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ABSTRACT

Background: *Panax ginseng* (ginseng) is a traditional medicine that is reported to have cardioprotective effects; ginsenosides are the major bioactive compounds in the ginseng root.
Methods: Magnetic molecularly imprinted polymer (MMIP) nanoparticles might be useful for both the extraction of the targeted (imprinted) molecules, and for the delivery of those molecules to cells. In this work, plant growth regulators were used to enhance the adventitious rooting of ginseng root callus; imprinted polymeric particles were synthesized for the extraction of ginsenoside Rb_1 from root extracts, and then employed for subsequent particle-mediated delivery to cardiomyocytes to mitigate hypoxia/reoxygenation injury.
Results: These synthesized composite nanoparticles were first characterized by their specific surface area, adsorption capacity, and magnetization, and then used for the extraction of ginsenoside Rb_1 from a crude extract of ginseng roots. The ginsenoside-loaded MMIPs were then shown to have protective effects on mitochondrial membrane potential and cellular viability for H9c2 cells treated with $CoCl_2$ to mimic hypoxia injury. The protective effect of the ginsenosides was assessed by staining with JC-1 dye to monitor the mitochondrial membrane potential.
Conclusion: MMIPs can play a dual role in both the extraction and cellular delivery of therapeutic ginsenosides.

1. Introduction

Myocardial ischemia/reperfusion injury is the major pathophysiology of cardiovascular disease (CVD) [1]. The Global Burden of Disease project predicts that CVD is likely to remain the leading cause of death for the foreseeable future [2]. Oxidative stress injury and mitochondria-mediated apoptosis plays a key role during myocardial ischemia-reperfusion injury [3]. Identification of natural antioxidants such as herbs is one important strategy to develop safe and effective anti-myocardial ischemia-reperfusion injury drugs [4]. Ginsenosides are natural product steroid glycosides and triterpene saponins, and found almost exclusively in the plant genus *Panax* (ginseng) [5]. They have been shown to have

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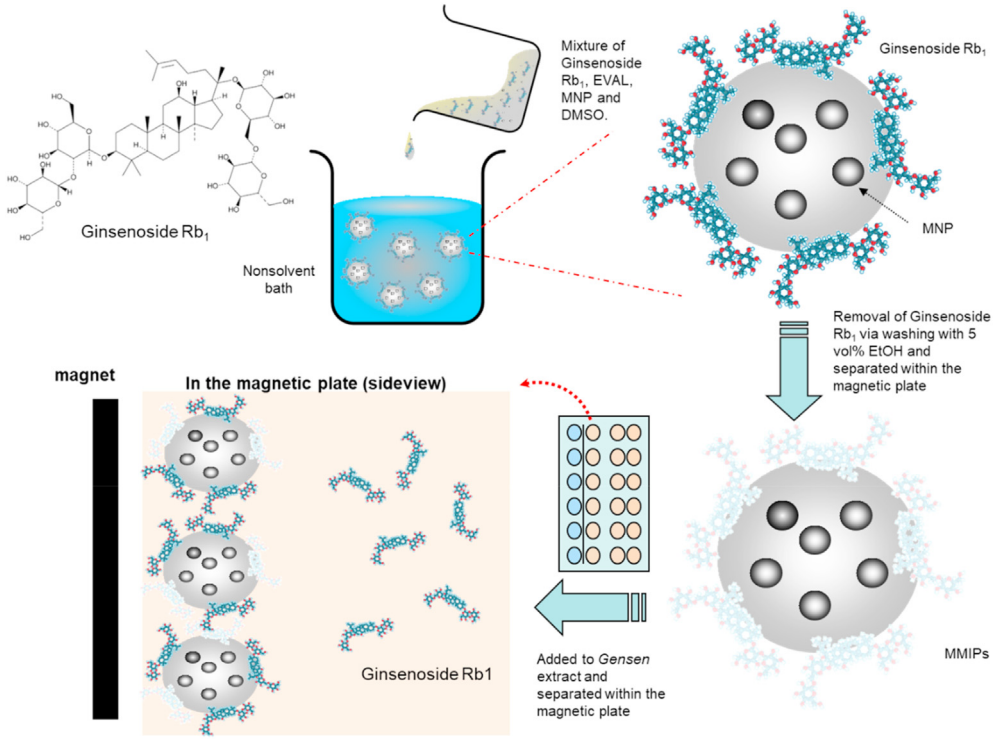

Scheme 1. The preparation of magnetic ginsenoside Rb_1-imprinted EVAL nanoparticles (MMIPs) and their use for the extraction of ginsenoside Rb_1 from ginseng root extract.

numerous and varied therapeutic uses [6–12]. Of special relevance to this work, ginsenoside Rb_1 exerts antioxidant functions and thus has protective effects on human umbilical vein endothelial cells *in vitro* [13], and can prevent the ischemic brain damage or ischemic injury to spiral ganglion cells by scavenging free radicals [14–17]. Ginsenoside Rb_1 was found to provide myocardial protection by inducing an estrogen receptor-dependent crosstalk among the Akt, JNK, and ERK 1/2 pathways to prevent injury to H9c2 cardiomyocytes and apoptosis induced by hypoxia-reoxygenation [18]. Moreover, this protects the mitochondria by reducing the release of cytochrome *c* and the expression of cleaved-caspase-3 in the cytoplasm, ultimately reducing apoptosis [19].

Molecularly imprinted polymers (MIPs) have been developed for detection, separation, purification, and delivery of bioactive molecules [20,21]. Encapsulation of magnetic nanoparticles (MNPs) into MIPs is often used to facilitate separation by magnetic fields [20,22,23]. The use of molecular fragments as templates or epitopes for the recognition of larger molecules is of great current interest [20], but when the whole molecule is relatively inexpensive, it is preferable to that as the template, and this is the approach we take here [21].

In this work, ginseng root growth from explants was induced, and the roots were then extracted with ethanol. Magnetic molecularly imprinted polymers (MMIPs) were prepared with magnetic nanoparticles, poly (ethylene-*co*-vinyl alcohol)s, EVALs, and templates via phase separation of a polymer solution in dimethyl sulfoxide (DMSO) on introduction into a non-solvent. The synthesized MMIPs were then characterized by dynamic light scattering to determine size distribution, and by atomic force microscopy to examine surface morphology. The binding capacity and the extraction of ginsenosides with MMIPs was then measured by high performance liquid chromatography (HPLC). The viability of H9c2 cardiomyocytes, after treatment with $CoCl_2$ to induce hypoxia, was measured after incubation with ginseng root extract or ginsenoside-loaded MMIPs. Finally, the mitochondrial membrane potential in hypoxic myocytes was monitored by the staining of JC-1 aggregates or monomers.

2. Experimental

2.1. Reagents and chemicals

Ginsenosides obtained from ChromaDex Inc (CA, USA). Poly(ethylene-*co*-vinyl alcohol) (EVAL), iron (III) chloride 6-hydrate (97%), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and JC-1 mitochondria staining kit For mitochondrial potential changes detection were from Sigma-Aldrich Co. (St. Louis, MO). 1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) was from Wako Pure Chemical Industries, Ltd. (Osako, Japan). Iron (II) sulphate 7-hydrate (99.0%), hydrochloric acid and sodium hypochlorite were from Panreac (Barcelona, Spain). Acetonitrile and methanol high purity (>99.9%) for HPLC grade were from Fisher Scientific (NJ, U.S.A.) and Aencore Chemical PTY., LTD. (Surrey Hills, Australia), respectively. Dimethyl sulfoxide (DMSO, purity>99.9%) was ordered from J. T. Baker Chemical Co., (Phillipsburg, NJ). Lanosterol was obtained from Tokyo Chemical Industry Co., LTD (Japan). Gibco Dulbecco's modified Eagle medium and nutrient mixture F-12 (DMEM/F-12) were purchased from *Life Technologies Corporation* by Gibco company. Cobalt (II) chloride hexahydrate, (purity 98%) was from Alfa Aesar by Thermo Fisher Scientific. All chemicals were used as received unless otherwise mentioned.

Table 1
The effect of (A) 2,4-D, (B) NAA and (C) NAA and TDZ on adventitious root formation from callus of *Panax ginseng*. Data evaluation in all the experiments was according to analysis of variance (ANOVA), and the significant differences among the treatments were compared using the Duncan's multiple range test (DMRT) with a 0.05 level of probability [28].

Table 1A

2,4-D (mg/L)	Percentage of rooting (%)*	Number of roots	Length of the longest root (cm)
0	0	–	–
2.0	5	4.5 ± 1.5	0.45 ± 0.15
4.0	0	–	–
6.0	0	–	–
8.0	0	–	–
10.0	0	–	–

Table 1B

NAA (mg/L)	Percentage of rooting (%)*	Number of roots	Length of the longest root (cm)
0	0	–	–
2.0	0	–	–
4.0	40.0	11.0 ± 0.5^{a}**	1.85 ± 0.40^{a}
6.0	35.0	6.3 ± 0.8^{b}	0.94 ± 0.24^{b}
8.0	10.0	2.3 ± 1.4^{c}	0.88 ± 0.14^{b}
10.0	10.0	1.0± 0^{c}	0.28 ± 0.26^{c}

Table 1C

NAA (mg/L)	TDZ (mg/L)	Percentage of rooting (%)*	Number of roots	Length of the longest root (cm)
0	0	0	–	
10.0	0.01	75.0	2.5 ± 1.0^{b}**	0.65 ± 0.23^{a}
10.0	0.1	30.0	6.3 ± 0.8^{a}	0.73 ± 0.23^{a}
1.0	1.0	0	–	
0.1	10.0	0	–	
0.01	10.0	0	–	

*Twenty replicates were tested for each treatment.
**According to DMRT ($P \leq 0.05$), the means ± standard errors within a column that followed by the same letter were regarded as not significantly different.

2.2. Induction of adventitious roots from ginseng callus

Six-month-old ginseng calluses (each approximately 0.1 g) were tested for the ability to form adventitious roots in the presence of plant growth retardants (PGRs), including 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), combinations of NAA and TDZ. All the calluses were cultured in an incubator with a 16/8 h (light/dark) photoperiod at an irradiance of 42–55 μmol m^{-2} s^{-1} and a temperature of 25 ± 2 °C. Twenty replicates were prepared for each PGR combination. The percentage of rooting, number of roots and length of the longest root were scored for four rooting calluses in each treatment, after three months of culture.

2.3. Extraction of ginsenosides from the extract of ginseng roots

The formation (see Scheme 1) and characterization of magnetic ginsenoside Rb_1-imprinted EVAL nanoparticles can be found in the *Electronic Supplementary Information* (ESI) **S1.1 and S1.2.** respectively. Adventitious roots of ginseng from callus were dried in a 50 °C oven for 24 h. The dried roots were soaked in 95% ethanol for one week at room temperature, at weight ratio 1:50. Then the root and ethanol mixture were sonicated using a 200 W ultrasonic cleaning tank for 3 h. The mixture was then heated to 80 °C using an efflux condensing tube for 1 h, Next, the ethanol was removed using rotary evaporation (IKA RV10, Germany) at 1000 mbar (0.987 atm) and 20 rpm for 1 h. The concentrated ginsenoside was collected after centrifugation at 5000 rpm for 5 min, and examined by HPLC. This ginsenoside stock solution (250 μL) was then exacted with 100 μg of MMIPs (Rb_1 as template) particles in an eppendorf for 30 min without mixing, and then particles were collected using a magnetic plate. Next, 5 vol% of ethanol 250 μL was added into the collected MMIPs for the elution of ginsenosides from MMIPs using the automatic working mode of a vortex mixer for 10 min and then the extraction solution was obtained using a magnetic plate. The adsorption of Rg_1 and Rb_1 from the extract of ginseng roots with MMIPs, stock and remaining were analyzed by HPLC as described in the *Electronic Supplementary Information* (ESI) **S1.3**.

2.4. Cell culture and hypoxia/reoxygenation treatment

The H9c2 cardiomyocyte cell line from rat heart myoblast was purchased from the Bioresource Collection and Research Center (BCRC; Taipei, Taiwan). The control groups of H9c2 cells were cultured in DMEM medium of high glucose with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 atmosphere without $CoCl_2$. The hypoxia-reoxygenation of H9c2 cells were grown in DMEM/F12 medium without glucose at 37 °C and 5% CO_2 atmosphere using 30 μg/mL $CoCl_2$ for 24 h. For all experiments, H9c2 cells of 1×10^4 per well were seeded in 24-well plates for 24 h.

2.5. Cell viability and JC-1 staining

The H9c2 cells (1×10^4 cells) were seeded in 24-well plates and treated with/without 4 μM $CoCl_2$ in DMEM/F12 medium at 37 °C and 5% CO_2 for 24 h on the 1st day. Cobalt chloride ($CoCl_2$) induces chemical hypoxia of H9c2 cardiomyocytes [24,25]. The Cell Counting Kit-8(CCK-8) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used to measure cytotoxicity of $CoCl_2$ with and without MNIPs/ginsenosides-loaded MMIPs. Medium was removed from each well, and then 50 μL CCK-8 and 450 μL DMEM was added in each well on the 2nd day (1 day after seeding.) The 24-well plate was incubated for a further 3 h at 37 °C before ELISA measurements. The absorption intensities were measured at 450 nm (I_{450}) and the reference absorption (I_{ref}) at 650 nm by an ELISA reader (CLARIOstar, BMG Labtech, Offenburg, Germany). The cellular viability (%) was then calculated from the ratio of effective absorption (I_{450}–I_{ref})

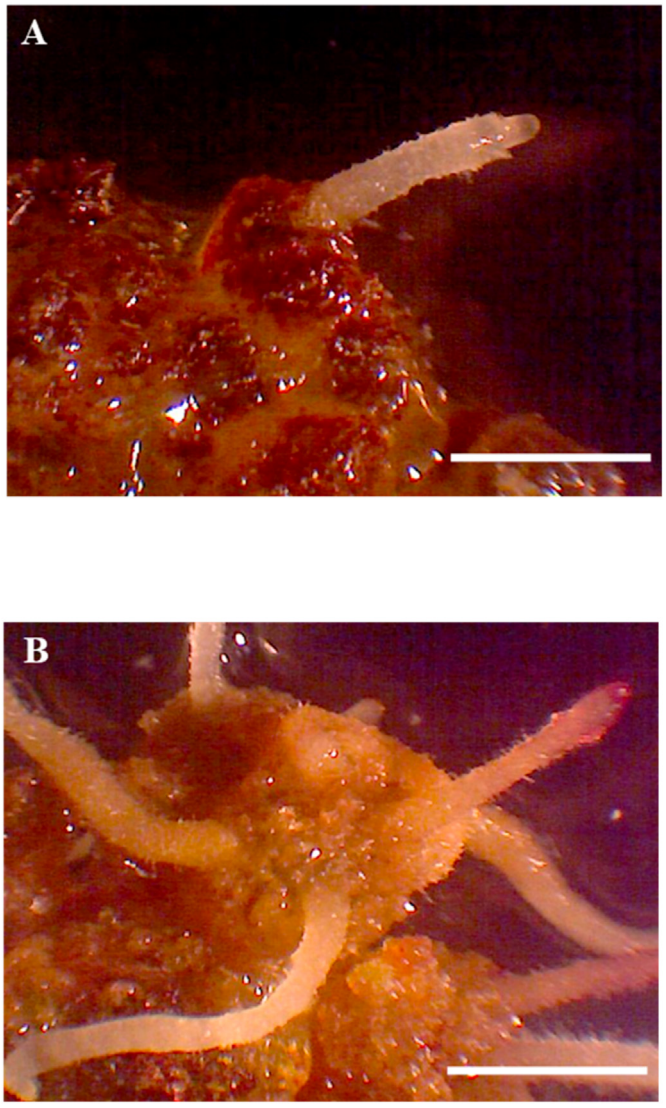

Fig. 1. Adventitious root formation from callus of *Panax ginseng*. (A) Root formation from callus after one month of culture (Bar = 0.5 cm). (B) Root formation from callus after two months of culture (Bar = 0.2 cm).

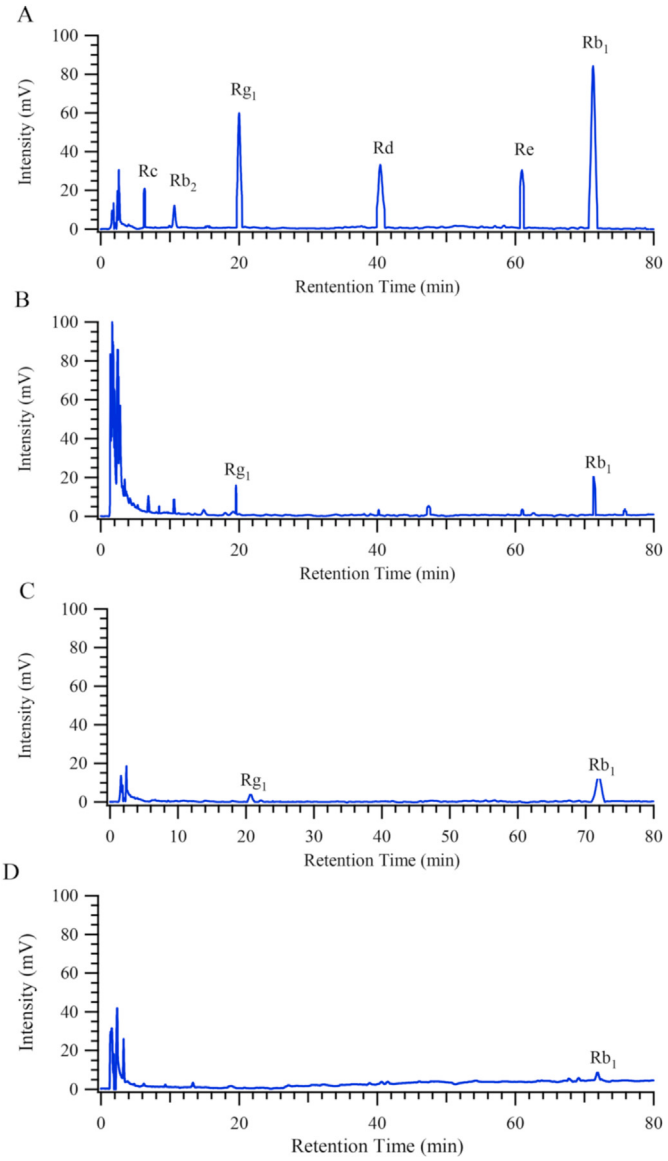

Fig. 2. HPLC retention of (A) ginsenoside standards; (B) extract of ginseng roots; further extraction with (C) MMIPs or (D) MNIPs.

of experimental cells to controls. The CCK-8 solutions were removed. The ginsenosides were exacted from the concentrated ginsenoside solution (250 μL) using 100 μg MMIP as described in section 2.3. The bound ginsenosides on MMIPs were washed off and measured using HPLC (the amount of bound ginsenoside Rb_1 was 51.5 mg/g MMIPs).

The ginsenoside-loaded MMIPs were collected using a magnetic plate, and then added to DMEM medium to obtain a final concentration of 100 μM of (MMIP-bound) ginsenoside Rb_1. Then 500 μL of the ginsenoside-loaded MMIPs/DMEM solution was added to each well on 24 well plate, and then incubated 24 h. The ginsenoside-loaded MMIPs/DMEM solution was replaced every day with fresh MMIP/ginsenoside solution. The cellular viability (%) was obtained using CCK-8 test on the 2nd and 5th day. For JC-1 staining, culture medium was removed and cells were washed with 0.5 mL PBS for each well. The 400 μL JC-1 solution was added and cells were incubated for 20 min at 37 °C. The JC-1 agent was removed and the cells were washed twice with DMEM medium. The stained cells were observed by fluorescence microscopy and analyzed on a fluorescent microplate reader (CLARIOstar, BMG Labtech, Offenburg, Germany). The fluorimeter excitation is 525 nm and 490 nm for JC-1 aggregates and monomer, respectively. The fluorimeter emission wavelengths are 590 nm and 530 nm.

3. Results and discussion

Several treatments of plant growth regulators (PGRs) were found to induce adventitious rooting from callus of *P. ginseng*, Table 1. PGR 2,4-D gave a low percentage of rooting from callus of ginseng at 2.0 mg/L, and gave no response at higher concentrations (Table 1A); thus, 2,4-D (alone) was not suitable for inducing re-differentiation in *P. ginseng*. NAA, used alone, could induce rooting of ginseng from calluses at concentrations of 4.0 mg/L or higher (Table 1B), though the highest percentage of rooting was found at 4.0 mg/L NAA. This, the lowest effective concentration, gave a significantly higher number of roots and significantly greater length of the longest root when compared to other NAA concentrations. The ginseng root formed from the peripheral region of the callus (Fig. 1A) and showed an unsynchronized development

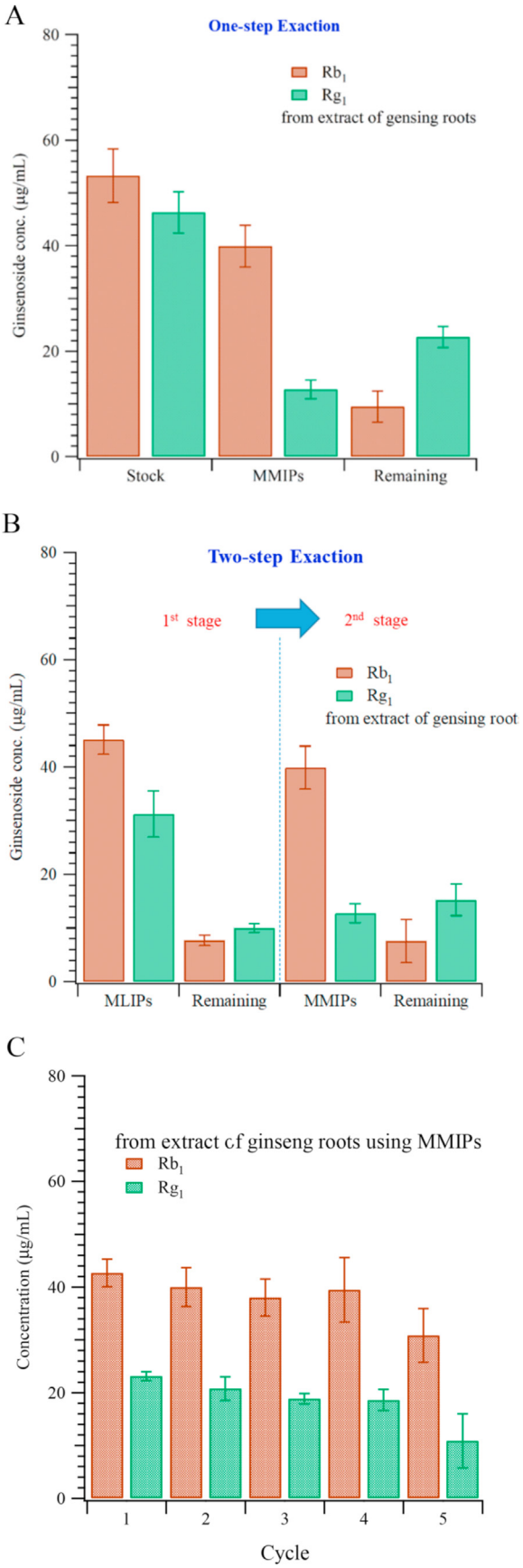

Fig. 3. Concentration of Rg_1 and Rb_1 in the extract of ginseng callus ("Stock"), recovered after extraction with MMIPs, and remaining in the stock after the extraction. (A) Single-step extraction using Rb_1-imprinted MMIPs; (B) two-step extraction using lanosterol-imprinted MMIPs in the first step. (C) The reusability of the MMIPs, showing good reproducibility for four cycles.

(Fig. 1B). Auxin/cytokinin ratios are thought to control plant *in vitro* morphogenesis, with higher ratios promoting rooting, and lower ratios promoting shooting [26]. Therefore, TDZ was used to modulate the auxin/cytokinin ratios and thus induce rooting of ginseng. Surprisingly, it was found that only when the concentration of NAA was higher than TDZ could rooting be induced from *P. ginseng* callus (Table 1C). Among the series of combinations of NAA and TDZ, the highest rooting percentage (75%) was found at 10 mg/L NAA combined with 0.01 mg/L TDZ, as shown in Table 1C. The higher TDZ concentration (0.1 mg/L) increased the number of roots approximately six-fold, but with a significantly decreased percentage of rooting (30% vs 75%) of ginseng callus. NAA, used alone, at concentrations of 4.0 mg/L was used for subsequent work.

Magnetic molecularly imprinted polymer particles were synthesized using ginsenoside Rb_1 as described in the Experimental section, above. The characterization of MMIPs is shown in Fig. S2 and discussed in the *Electronic Supplementary Information* (ESI S2). HPLC was used to quantify ginsenoside binding to the MMIPs. Fig. 2A shows HPLC of a set of ginsenoside standards; ginsenosides Rc, Rb_2, Rg_1, Rd, Re and Rb_1 were eluted at 6.35, 10.65, 20.01, 40.42, 60.96 and 71.22 min. The crude extract of dried ginseng roots was found to have almost all of them, Fig. 2B, but has higher concentrations of ginsenosides Rg_1 and Rb_1 at 50–60 mg/mL (*vide infra*, Fig. 3A). Fig. 2C shows the HPLC of the extract washed off MMIPs, indicating the presence of ginsenosides Rg_1 and Rb_1. In contrast, the eluate from the MNIPs were found to contain only a small amount of ginsenoside Rb_1 (from non-specific binding); Fig. 2D.

The results of the one-step extraction method (using only MMIPs for the extraction from ginseng root stock) is shown in Fig. 3A. The bound ginsenosides Rg_1 and Rb_1 were washed off from MMIPs (templated with Rb_1) with vortex mixing for 10 min, recovering about 74.9 ± 7.5% and 27.5 ± 3.8% of the stock concentrations, respectively. 17.8 ± 5.5% of Rb_1 and 53.0 ± 3.8% of Rg_1 were found to remain in the stock; the remainder represents bound ginsenosides that could not be washed off. A two-step extraction method was also studied, in which MLIPs (using lanosterol, a ginsenoside analogue, as template) were employed for the first extraction step, and then the wash was further extracted with ginsenoside MMIPs. Results are shown in Fig. 3B. Interestingly, imprinting with the ginsenoside Rb_1 gives about 80% of the efficiency obtained using lanosterol as the template molecule [27]. The final extracted amount of the ginsenoside Rb_1 using the MMIPs (i.e. ginsenoside Rb_1 as the template) is almost the same in two-step extraction; however, the extracted amount of Rg_1 was dramatically reduced. Fig. 3C shows the reusability of the MMIPs through five cycles. There was a noticeable deterioration in performance in the fifth cycle, but performance remained good through the first four.

In this study, cardiomyocytes were treated with apoptosis-inducing cobalt chloride ($CoCl_2$), and then JC-1 was used to monitor mitochondrial health and recovery, with or without the delivery of ginsenosides. Fig. 4A–D shows the staining of H9c2 cells with JC-1 dye. Fig. 4A and B are controls showing H9c2 cells treated with 30 μg/mL of $CoCl_2$; the green and red indicate the monomeric and aggregated JC-1, respectively. Healthy mitochondrial membrane potential is accompanied by a larger proportion of JC-1 in the aggregated, red form. Mitochondrial membrane potential was decreased with the $CoCl_2$ treatment, and more monomeric JC-1 (green) was found. After the administration of the MMIPs or the MNIPs with ginseng root extract, cells were stained with JC-1 on the

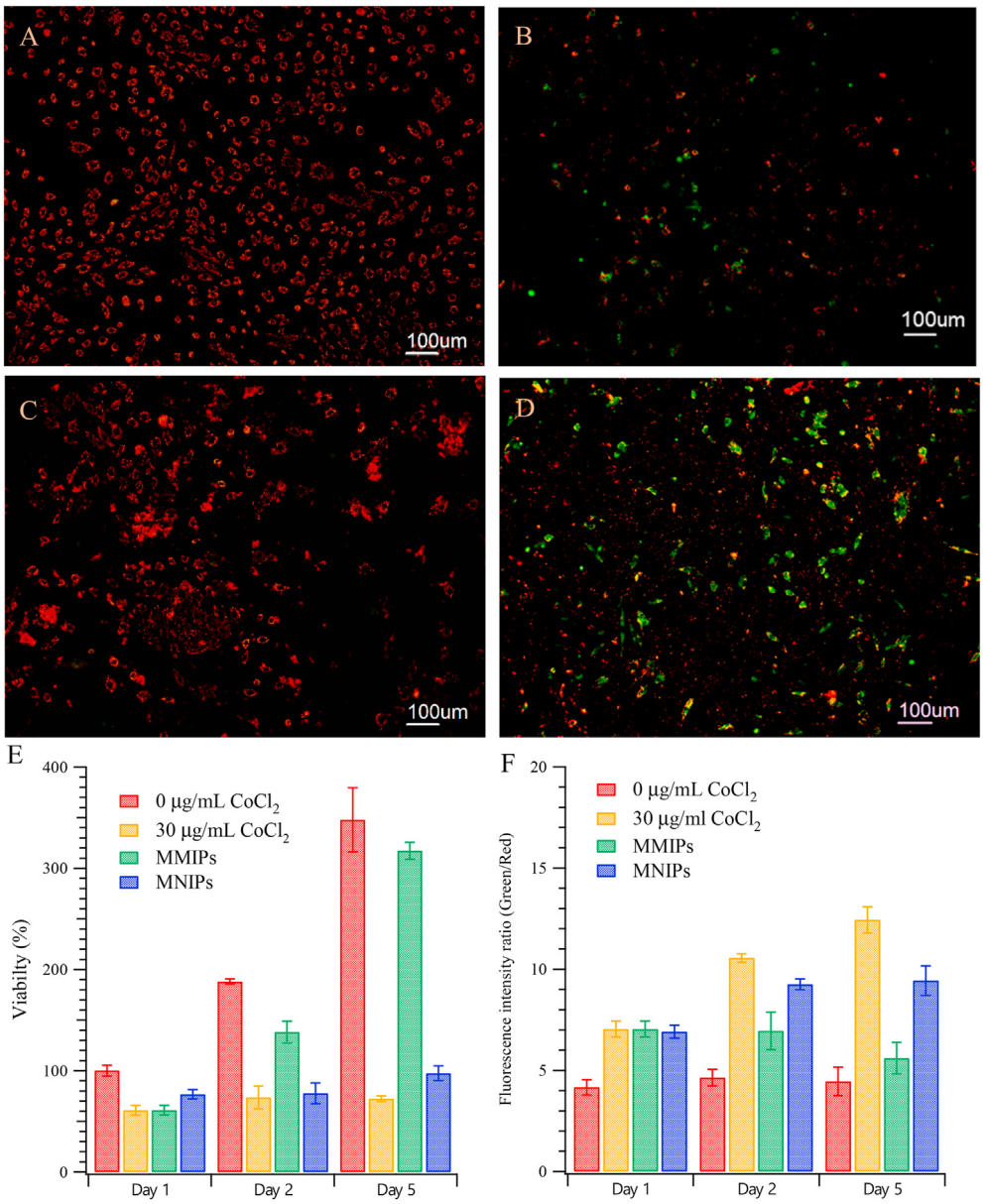

Fig. 4. Images of cell apoptosis of (A) H9c2 cells, which were (B) pre-treated with 30 mg/mL $CoCl_2$ and then with added (C) MMIPs; (D) MNIPs, both with bound ginsenosides from ginseng root extract. (E) Cellular viability and (F) mitochondrial damage (as assessed by the JC-1 green: red ratio) in H9c2 cells in the abovementioned conditions.

2nd day, shown in Fig. 4C and D, respectively. Cardiomyocytes treated with ginsenoside-loaded MMIPs showed healthier mitochondria (more JC-1 aggregates, Fig. 4C), while cells treated with MNIPs showed less recovery, Fig. 4D. The cellular viability (percentage of live cells, normalized to day 0) is shown in Fig. 4E. Viability after adding $CoCl_2$ was about 60% on the first day, with very small increases in cell numbers over the following days. When MMIPs with ginseng roots extract were added on the 2nd day, cell growth largely recovered, with about 91% of the number of live cells (compared with controls) on the 5th day. The non-imprinted nanoparticles had a much weaker (though still beneficial) effect, likely owing to non-specific binding of ginsenosides. The ratio of JC-1 monomer fluorescence (green) to aggregate fluorescence (red) is plotted in Fig. 4F. This figure shows clearly the protective effect of MMIPs with bound ginsenosides on mitochondrial membrane potential in H9c2 cells; on day 5, the membrane potential, as assayed by the fluorescence ratio, is nearly the same for cells treated with $CoCl_2$ and MMIPs as for healthy control cells. As with viability, non-imprinted MNIPs had only slight beneficial effects.

4. Conclusions

The extraction and cellular delivery of bioactive molecules (e.g. ginsenosides) are of great interest in targeted cellular therapies. Conventional approaches to extraction of bioactive molecules may require toxic organic solvents or expensive or time-consuming concentration procedures. Extraction with molecularly imprinted polymers may overcome both problems; encapsulation of magnetic nanoparticles further facilitates extraction and manipulation of MIPs. This work demonstrated not only the induction of adventitious roots from ginseng callus, but also the effective imprinting of ginsenoside Rb_1 into magnetic nanoparticles (MMIPs), and showed that the particles could be used for the extraction of ginsenosides from ginseng root extracts. The ginsenoside-loaded MMIPs were

then shown to have protective effects on mitochondrial membrane potential and cellular viability for H9c2 cells treated with $CoCl_2$ to mimic hypoxia injury. These encouraging results suggest that extraction and delivery of ginsenosides using MIPs may have important therapeutic and healthcare applications.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.01.005.

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