Synthesis and Characterization of Magnesium Gluconate Contained Poly(Lactic-Co-Glycolic Acid)/Chitosan Microspheres

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Abstract

The goal of the present study was to fabricate and investigate the chitosan coated Poly (lactic-co-glycolic acid) (PLGA) microspheres for the development of controlled release magnesium delivery system. PLGA based microspheres are ideal vehicles for many controlled release drug delivery applications. Chitosan is a naturally occurring biodegradable and biocompatible polysaccharide, which can coat the surface of PLGA to alter the release of drugs. Magnesium gluconate (MgG) was encapsulated in the PLGA and PLGA/chitosan microspheres by utilizing the double emulsion solvent evaporation technique for controlled release study. The microspheres were tested with respect to several physicochemical and biological properties, including morphology, chemical structure, chitosan adsorption efficiency, magnesium encapsulation efficiency, *in vitro* release of magnesium ions, and cellular compatibility using both human adipose-derived stem cells (ASCs) and PC12 cells. Chitosan coated PLGA microspheres can significantly control the release of magnesium ions compared to uncoated PLGA microspheres. Both coated and uncoated microspheres showed good cellular compatibility.

Keywords

Magnesium deficiency, Drug delivery, Biodegradable polymers, Surface coating, Controlled release

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1. Introduction

Magnesium (Mg) is the second most abundant intracellular element in the human body. Magnesium is associated with more than 300 biochemical reactions and is an indispensable element for energy metabolism, protein synthesis and DNA replication. Magnesium helps to maintain normal muscle and nerve function, supports healthy immune system, and promotes normal blood pressure. Magnesium deficiency is a common problem in 7-11% of hospitalized patients [1]. Magnesium deficiency has been associated with critical health issues, including congestive heart failure, sudden cardiac death, myocardial infarction, diabetes, hypertension, coronary heart disease and osteoporosis [2-5]. Patients with Mg deficiency are usually treated by magnesium administration [6,7]. Hypermagnesemia is commonly occurred due to excessive administration of magnesium, especially in patients with reduced renal function [1,8,9]. Hypermagnesemia is associated with a number of health issues, including hypotension, cardiac arrest, neuromuscular disorder, bradycardia and paralytic ileus [9-12]. Furthermore, magnesium uptake by cells is slow and it requires sustained correction of Mg deficiency [9].

Controlled release drug delivery systems offer numerous advantages over traditional methods of drug delivery by eliminating the need of repetitive drug administration with predetermined release rates and increase of the efficacy of the drugs [13,14]. Polymeric microspheres are ideal vehicles for many controlled delivery applications due to their biocompatibility, high bioavailability, ability to encapsulate a variety of drugs and sustained drug release characteristics [15].

Poly(lactic-*co*-glycolic acid) (PLGA) is a biodegradable, biocompatible and, Food and Drug Administration (FDA) approved synthetic polymer that exhibits a wide range of erosion times

[16]. Poly(lactic-*co*-glycolic acid) has been extensively studied for the development of devices for controlled delivery of therapeutic drugs, proteins, genes and other macromolecules. In these applications, surface of the PLGA is typically protected by poly(ethylene glycol) (PEG) for prolonged circulation and enhanced cellular uptake [17]. However, PEG can interfere with the interactions between drug carriers and target cells, and negatively influences the therapeutic outcomes [18].

Chitosan is a cationic polysaccharide produced by the exhaustive deacetylation of chitin, a structural element in the exoskeleton of crustaceans and insects. The primary amino groups of the chitosan are responsible for several important properties, such as mucoadhesion, *in situ* gelation, transfection, permeation enhancement and efflux pump inhibitory properties. Chitosan has recently sparked interest in the field of surface modification due to several desirable properties: minimal foreign body reaction, mild processing conditions, chemical reactivity and cost effectiveness [18,19].

This study was designed to investigate the feasibility of controlled release of magnesium ions from chitosan coated and uncoated PLGA microspheres. We used magnesium gluconate (MgG) as a model compound to study the release kinetics of magnesium ions from the microspheres. We hypothesized that chitosan coating on the surface of PLGA could be used to significantly control the release of magnesium ions. MgG is a therapeutic drug used to treat Mg deficiency [10]. In recent studies, MgG has exhibited the highest level of bioavailability among different magnesium salts and has been recommended as the optimal salt for treatment of Mg deficiency [20,21]. For controlled release study, MgG encapsulated PLGA and PLGA/chitosan microspheres were prepared by utilizing the double emulsion solvent evaporation technique. Towards the potential use of these microspheres *in vivo* application, several physiochemical

properties, such as morphology, chemical structure, chitosan adsorption efficiency, magnesium encapsulation efficiency and release of magnesium ions were studied. Cellular compatibility of these microspheres was studied with human adipose-derived stem cells (ASCs) and PC12 cells.

2. Materials and Methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (PLGA, lactic acid/glycolic acid = 50:50, with carboxylate end group, inherent viscosity 0.15-0.25) was purchased from Durect Corporation (Birmingham, AL, USA). Chitosan (Mw 2.5 kDa) was purchased from Creative PEGWorks (Winston Salem, NC, USA). Magnesium gluconate dihydrate (MgG, Mw 322.64, 98%) was purchased from Pfaltz & Bauer (Waterbury, CT, USA). Poly(vinyl alcohol) (PVA, ~ 99% hydrolyzed), ninhydrin, hydrindantin, lithium acetate dihydrate and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dichloromethane (DCM, ~ 99%), glacial acetic acid and sodium hydroxide (NaOH) were purchased from Acros Organics (Morris Plains, NJ, USA). Dimethyl sulfoxide (DMSO, ~ 95%), phosphate buffered saline (PBS), ethanol (92-93%) and hydrochloric acid (HCl, 35-38%) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Fabrication of microspheres

PLGA/chitosan microspheres were fabricated by utilizing the double emulsion solvent evaporation technique with some modifications [16]. PLGA (1 g) was dissolved in 4 ml of DCM. The PLGA solution was added to 2 ml of 1% PVA solution and emulsified on ice bath for 2 min with Sonic Dismembrator (Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 50% amplitude and on a 10 sec on/off pulse mode. Chitosan was dissolved in 20 ml of deionized water (DI water, pH

5) and transferred to 80 ml of DI water containing 0.1% PVA. The emulsified PLGA solution was added to the chitosan solution dropwise using Single Syringe Infusion Pump (Cole-Parmer, Vernon Hills, IL, USA) and stirred overnight for DCM evaporation, particle hardening and chitosan adsorption to the surface of PLGA. Chitosan coated PLGA microspheres were collected by Sorvall Stratos Centrifuge (Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 6000 rpm and 4 °C for 30 min. The microspheres were washed three times with DI water. The purified PLGA/chitosan microspheres were lyophilized for two days by using Freezone Freeze Dryer (Labconco, Kansas City, MO, USA).

For encapsulation study, MgG was dissolved in 8 ml of DI water and mixed with 1 g of PLGA dissolved in 4 ml of DCM. The combined mixture was emulsified in the same way as described above. Ultrasonic emulsification method was carried out for the entrapment of ionized magnesium into the polymer networks of PLGA. The control microspheres of PLGA and PLGA/MgG were fabricated by adding respective emulsified solutions drop wise to 80 ml of DI water containing 0.1% PVA.

2.3. Assessment of morphology and microsphere size distribution

The morphology of the microspheres was observed by scanning electron microscope (SEM) (Hitachi SU8000, Japan). Double sided carbon tape was used for attaching the specimens to the sample holder. Prior to imaging with the SEM, samples were gold sputtered under vacuum by using Polaron SEM Coating System (Quorum Technologies, East Sussex, UK) for 1 min. The samples were imaged at an accelerating voltage of 10 kV and current of 5 μ A.

The size distribution of the microspheres was determined through SEM images with the use of ImageJ software (NIH, USA). First, length of each scale bar was measured in pixels. 75

microspheres of each group ($n = 3$) were also measured in pixels. The number of pixels was converted to μm using scale factor. Finally, the average size and standard deviation were calculated based on converted ImageJ data.

2.4. Chemical structure analysis

Fourier Transform Infrared Spectroscopy (FTIR) was used to analyze the chemistry between PLGA and chitosan. FTIR spectra were recorded at 200 scans using IR Prestige21 FTIR Spectrometer (Shimadzu, Kyoto, Japan). The spectra were calculated from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} . Prior to obtaining the FTIR spectra, each sample was ground and dispersed in KBr matrix. A pellet was then formed by compressing the sample at high pressure. The pellet was placed in the FTIR holder to perform the measurement at absorbance mode.

2.5. Determination of chitosan adsorption efficiency and in vitro dissolution study

Chitosan adsorption efficiency and dissolution study were carried out by using the ninhydrin assay according to the previously reported method [22]. Briefly, 10 ml of lithium acetate buffer was prepared by dissolving 4.08 g of lithium acetate dihydrate in 6 ml of DI water. The pH of the resulting solution was adjusted to 5.2 using glacial acetic acid and NaOH, and the volume adjusted to 10 ml with DI water. The ninhydrin reagent was prepared by adding 10 ml freshly prepared lithium acetate buffer to 0.8 g ninhydrin and 0.12 g hydrindantin in 30 ml of DMSO. Ninhydrin reagent (0.5 ml) was added to 0.5 ml of sample solution in a glass scintillation vial. The vial was immediately capped, briefly shaken by hand and heated in boiling water for 30 min. After cooling, 15 ml of 50% ethanol was added to the vial. The vial was then vortexed using Analog Vortex Mixer (Thermo Fisher Scientific, Fair Lawn, NJ, USA) for 30 sec in order to oxidize the excess of hydrindantin. The absorbance of each solution was measured by using

GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 570 nm. The concentration of chitosan in the sample was calculated from a standard calibration curve.

For chitosan adsorption efficiency, six PLGA/chitosan samples were prepared by varying the chitosan content from 0.1 g to 0.6 g for 1 g of PLGA. Ninhydrin reagent (0.5 ml) was mixed with 0.5 ml of PBS (pH 7.4) containing 50 µg of each sample. For chitosan dissolution study, 0.1 g of optimized PLGA/chitosan sample was added to 100 ml of PBS. The mixing solution was then incubated in Dubnoff Metabolic Shaking Incubator (Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 37 °C and 30 rpm. At different time intervals, aliquots of 0.5 ml were collected from the sample solution and added to 0.5 ml of ninhydrin reagent in a glass scintillation vial for analysis using the ninhydrin assay in the same way as described above.

2.6. Determination of magnesium encapsulation efficiency and in vitro release study

Magnesium encapsulation efficiency and release study were carried out by using the Magnesium Colorimetric Xylidyl Blue Assay (Stanbio Laboratory, Boerne, TX, USA). 10 µl of sample solution was added to 1 ml of magnesium reagent in a cuvette. After 60 min, the absorbance of each solution was measured by GENESYS 10S UV-VIS Spectrophotometer at 520 nm. EDTA solution (6.25 g/l) was prepared. 10 µl of EDTA solution was added to each cuvette and absorbance was measured again at 520 nm after 30 min to be absolutely sure of the assay and to account for any cloudiness in the medium. The concentration of magnesium in the sample was calculated from a standard calibration curve.

For determination of magnesium encapsulation efficiency, six PLGA/chitosan/MgG samples were prepared by varying the MgG content from 0.2 g to 1.2 g for 1 g of PLGA and 0.4 g of

chitosan. 0.1 g of each sample was added to 15 ml of PBS (pH 7.4). Sample solution was disintegrated by ultrasonic disruption for 20 min using Sonic Dismembrator at 50% amplitude and on a 50 sec on and 10 sec off pulse mode. The sample solution was then vigorously vortexed at 3200 rpm using Analog Vortex Mixer for 10 min. Finally, 10 μ l of each sample solution was added to 1 ml of Xylidyl Blue reagent in a cuvette.

For magnesium release study, 0.5 g of optimized PLGA/MgG and PLGA/chitosan/MgG sample was added to 75 ml of PBS. The mixing solution was incubated in Dubnoff Metabolic Shaking Incubator at 37 °C and 30 rpm. At different time intervals, aliquots of 10 μ l were collected from the sample solution and added to 1 ml of Xylidyl Blue reagent in a cuvette. The absorbance of mixing solutions and their corresponding concentrations were measured following the same way as described above.

2.7. PC12 cell culture

All contents for the cell culture media were purchased from Gibco/Invitrogen Corporation (Carlsbad, CA, USA). The rat pheochromocytoma cell line PC12 was purchased from ATCC (CRL-1721) (Manassas, VA, USA), and cells were cultured at 37 °C with 5% CO₂ in collagen coated T-75 tissue culture flasks. The cells were maintained in DMEM/F-12 media including 10% horse serum and 1000 IUml⁻¹ penicillin/1000 μ gml⁻¹ streptomycin, and allowed to attach for 24 hr at 37 °C in a humidified incubator with a 5% CO₂ atmosphere.

2.8. Human adipose-derived stem cell (ASC) isolation

Human ASCs were isolated from harvested abdominal subcutaneous adipose tissue from a non-diabetic female undergoing elective surgery (age: 37, body mass index: 25.8), as previously described [23]. Briefly, adipose tissue was first minced while submerged in a collagenase

solution (type II, Worthington Biochemical Corp, Lakewood, NJ, USA). Collagenase solution, a matrix metalloproteinase (MMP) was used to break down the fibrous tissue in the whole fat and release more ASCs for isolation. The tissue in the collagenase solution was then gently agitated at 37 °C, filtered to remove large debris, and centrifuged. The cellular pellet was resuspended in erythrocyte lysis buffer and centrifuged again. The resulting cellular pellet was plated and cultured on tissue treated flasks in ASC plating medium (DMEM/F12, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, dexamethasone).

2.9. Assessing cytotoxicity of microspheres

In vitro cytotoxicity tests with ASCs and PC12 cells were conducted in sterile 24 multi-well tissue culture plates with 0.4 µm pore size cell culture inserts (Corning, NY, USA) at approximately 100,000 cells/well (n = 3 each). Microspheres (5 mg/well) were placed into inserts in contact with cell/media solution. A control group was also seeded along with inserts to be used as a reference baseline. Cells were cultured with microspheres for 9 days. Aliquots of culture media were collected at 3, 6 and 9 days to assess the cytotoxicity via a colorimetric method to quantitatively measure the lactate dehydrogenase activity (QuantiChrom Lactate Dehydrogenase Kit, BioAssay Systems, Hayward, CA, USA).

The live/dead staining for fluorescence microscopy was conducted using the methods hereafter. ASCs were seeded on Nunc Thermanox Coverslips (13mm diameter). Microspheres (5 mg/well) were placed into inserts in contact with cell/media solution. Cell media was changed on the 3rd and 5th day. On the 6th day, cell media was removed and cells were rinsed with PBS. The Live/Dead Viability/Cytotoxicity Kit from Life Technologies (Grand Island, NY, USA) was

used to identify the live and dead cells using a fluorescent microscope (Nikon E300, Tokyo, Japan).

2.10. Fabrication yields and statistical analysis

The experimental yield was divided by the theoretical yield to determine the percentage of fabrication yield of each sample. Fabrication yields of PLGA/MgG, PLGA/chitosan and PLGA/chitosan/MgG were calculated once the amounts of chitosan and MgG were optimized in their respective combinations.

Data were analyzed for significance with OriginPro software (OriginLab, Northampton, MA, USA) using one-way analysis of variance (ANOVA). *Post hoc* Tukey's test was performed with ANOVA for multiple comparisons. The α -value was set to 0.05 and p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Morphology and size distribution of microspheres

Surface morphology and size distribution of microspheres were examined through SEM images. Fig. 1 and 2 show the surface morphology and size distribution of corresponding microspheres, respectively. The microspheres were found to be uniform with some deviations and nominal aggregation. The mean diameters of PLGA, PLGA/MgG, PLGA/chitosan and PLGA/chitosan/MgG microspheres were 1.2, 1.0, 1.1 and 1.1 μm , respectively.

Fig. 1. SEM images of microspheres: (A) PLGA, (B) PLGA/MgG, (C) PLGA/chitosan and (D) PLGA/chitosan/MgG. Scale bars are 5 μm . The insets show higher magnification images of each corresponding SEM image.

Fig. 2. Microsphere size distribution: (A) PLGA, (B) PLGA/MgG, (C) PLGA/chitosan and (D) PLGA/chitosan/MgG.

3.2. FTIR spectra of microspheres

The FTIR spectra were measured to confirm the chitosan adsorption to the surface of PLGA. Fig. 3 shows the FTIR spectra of PLGA, chitosan, PLGA/chitosan and PLGA/chitosan/MgG. In the chitosan spectrum, the intense peaks at 1545 and 1630 cm^{-1} confirmed the presence of amine and amide bonds in the chemical structure of chitosan. In the PLGA spectrum, the peak of C-O-C stretching is at 1175 cm^{-1} , C-H bend is at 1390-1440 cm^{-1} , C=O is at 1760 cm^{-1} and C-H stretch is at 3000 cm^{-1} . The broad signal is at 3200-3700 cm^{-1} for N-H and O-H stretch. From PLGA/chitosan and PLGA/chitosan/MgG spectra, the chitosan adsorption to the surface of PLGA was confirmed by two intense peaks at 1545 and 1630 cm^{-1} , corresponding to amine and amide bonds, and broad N-H signal of chitosan at 3200-3700 cm^{-1} .

Fig. 3. FTIR spectra of PLGA, chitosan, PLGA/chitosan and PLGA/chitosan/MgG.

3.3. Chitosan adsorption efficiency

In this study, chitosan was adsorbed to the surface of PLGA and formed physical coating. Fig. 4 shows the relationship between mass fraction of chitosan adsorbed to the surface of PLGA and theoretical mass fraction of chitosan used during fabrication. Theoretical mass fractions of chitosan in six samples were 9.1, 16.7, 23.1, 28.6, 33.3 and 42.9%. The mean mass fractions of chitosan adsorbed to the surface of PLGA were 6.2, 12.1, 16.3, 18.1, 18.3 and 18.75%, respectively ($n = 3$ each). First four samples with theoretical mass fraction range from 9.1 to 28.6% were significantly different from each other ($p < 0.05$). There was no significant difference between fourth, fifth and six samples ($p > 0.05$). 18.1 % of chitosan was adsorbed as mass fraction to the surface of PLGA with significant efficiency when we used 0.4 g of chitosan for 1 g of PLGA (theoretical mass fraction = 28.6%). Therefore, 0.4 g of chitosan for 1 g of PLGA was optimized amount in this study and chitosan adsorption efficiency was 55% for this amount.

Fig. 4. Determination of chitosan adsorption efficiency.

3.4. *In vitro* dissolution profile of chitosan

The chitosan dissolution profile for various time points at pH 7.4 and 37 °C is shown in Figure 5. The results were obtained in terms of cumulative concentration of chitosan and each data point represents mean $\pm$ standard deviation of three independent samples. At the beginning, the dissolution of chitosan was faster and dissolution became slow after 72 hours. After 168 hours (7 days), 46.1% of chitosan was dissolved. The chitosan dissolution profile qualifies chitosan as a useful surface coating of PLGA for prolonged circulation and controlled release of magnesium ions.

Fig. 5. *In vitro* dissolution of chitosan from the surface of PLGA at pH 7.4 and 37 °C.

3.5. *Magnesium encapsulation efficiency*

Fig. 6 shows the relationship between concentration of encapsulated magnesium and theoretical concentration of magnesium used during fabrication. The concentrations of

magnesium used during fabrication in six samples were 3.0, 5.2, 7.0, 8.4, 9.5 and 10.4 millimolar (mM). The mean concentrations of encapsulated magnesium found experimentally in six samples were 1.03, 2.09, 3.13, 4.27, 4.51 and 4.59 mM, respectively (n = 3 each). First four samples with theoretical Mg concentration range from 3.0 to 8.4 mM were significantly different from each other ($p < 0.05$). There was no significant difference between fourth, fifth and six samples ($p > 0.05$). In this experiment, 4.27 mM of magnesium was encapsulated in the microspheres with significant encapsulation efficiency when we used 0.8 g of MgG for 1 g of PLGA (theoretical concentration = 8.35 mM). Therefore, 0.8 g of MgG for 1 g of PLGA was optimized amount in the encapsulation study and encapsulation efficiency was 50.8% for this amount.

Fig. 6. Determination of magnesium encapsulation efficiency.

3.6. *In vitro* magnesium release profiles

Magnesium release profiles are shown in Fig. 7. The results were obtained in terms of cumulative concentration of magnesium ion and each data point represents mean $\pm$ standard

deviation of three independent samples. The release profiles of PLGA/MgG and PLGA/chitosan/MgG microspheres showed sustained release of magnesium ions. Initially, the release of magnesium ions was faster which was followed by relatively slow release from PLGA and PLGA/chitosan matrices. There was significant difference of magnesium release between PLGA/MgG and PLGA/chitosan/MgG microspheres ($p < 0.05$). After 168 hours (7 days), 3.09 mM (72.4%) of magnesium released from the uncoated PLGA/MgG microspheres, whereas 2.24 mM (52.5%) of magnesium released from the chitosan coated PLGA/MgG microspheres.

Fig. 7. *In vitro* release of magnesium at pH 7.4 and 37 °C.

3.7. Microsphere fabrication yields

The following yields (%) were achieved in this study: the yield (%) for PLGA was 48.7, the yield (%) for PLGA/MgG was 50.7, the yield (%) for PLGA/chitosan was 42.5 and the yield (%) for PLGA/chitosan/MgG was 31.6. Fig. 8 shows the fabrication yields of different microspheres.

Data are expressed as averages with standard deviations of three independently prepared samples. The yields were measured based on theoretical basis of 1 g PLGA, 0.4 g chitosan and 0.8 g MgG. The discrepancy between actual yield and theoretical yield are due to sample losses during chitosan adsorption, magnesium encapsulation, separation, and purification. Because of having two hydrophilic elements and high amount of aqueous solution, the yield (%) of PLGA/chitc

Fig. 8. Fabrications yields of microspheres: (A) PLGA, (B) PLGA/MgG, (C) PLGA/chitosan and (D) PLGA/chitosan/MgG.

3.8. Cytotoxic effects of microspheres

Cytotoxicity was calculated using the control group as a baseline for healthy cell culture as instructed in assay protocols. The PC12 cells maintain average cytotoxicity levels under 22% while in contact with microspheres (Fig. 9). Cytotoxicity levels did not surpass the 15%

cytotoxicity marker in the microspheres containing ASCs (Fig. 10), which indicates sufficient biocompatibility. For the ASCs, the cytotoxicity levels were minimal after 3 days in culture with microspheres. After 6 days, cytotoxicity levels went up to about 13.8% and stayed under 15% on the ninth day of culture. At each time point, there was no significant difference between three samples (PLGA/chitosan, PLGA/MgG and PLGA/chitosan/MgG) in culture with ASCs and PC12 cells ($p > 0.05$).

The representative live/dead staining images in Fig. 11 appear to display a significantly higher amount of live ASCs than dead ASCs on the sixth day of culture. This result shows ASCs with a considerable amount of viability and minimal death amongst all three groups of microspheres. The comparatively low amount of red cells correlate to the cytotoxicity results reported in Fig. 10.

Fig. 9. Cytotoxic effects of microspheres on PC12 cells for 3, 6 and 9 days.

Fig. 10. Cytotoxic effects of microspheres on ASCs for 3, 6 and 9 days.

Fig. 11. Live/dead staining images of ASCs for 6 days : (A) PLGA/MgG, (B) PLGA/chitosan and (C) PLGA/chitosan/MgG.

4. Discussion

During the past two decades, significant advances have been made in the development of biodegradable polymeric biomaterials. PLGA is most examined among the various available biodegradable polymers because of its long clinical experience, favorable degradation characteristics and possibilities for sustained drug delivery [16]. But the surface properties of PLGA, hydrophobicity and high degradation rate can render PLGA unacceptable for systemic drug delivery applications. Presence of methyl group in PLGA renders its hydrophobicity. The combination of hydrophilic and hydrophobic polymers is a strategy to effectively control the release of water-soluble drugs [24,25]. We designed this study to fabricate biodegradable magnesium delivery system based on the combination of hydrophobic and hydrophilic polymers.

While biodegradable polymeric microspheres are fabricated using various techniques, such as solvent evaporation technique, phase separation and spray drying, the double emulsion solvent evaporation technique is considered to be the most common method and best suited to encapsulate water soluble therapeutics [16,26]. In this study, the double emulsion solvent evaporation technique was modified and successfully utilized to fabricate the microspheres. The microspheres were found to be 1 μm in average diameter with nominal aggregation. The smooth surface of microspheres was attributed to the soluble nature of PLGA in DCM and, chitosan and MgG in water. The mixture of MgG in water and PLGA in DCM produced a homogenous dispersion. High molecular weight chitosan exhibits high viscosity and low solubility in aqueous solution, which can confine their applications. But low molecular weight chitosan has low viscosity and high solubility in aqueous solution at physiological pH. In this study, we used low molecular weight of chitosan (Mw 2.5 kDa) for surface modification. The slight differences in

size of four types of microspheres could be achieved due to several factors, including DCM evaporation rate, effect of PVA, temperature and phase dispersion.

Chitosan is widely used for biomedical applications, especially for drug delivery, tissue engineering and regenerative medicine. During circulation, chitosan breaks down to harmless amino sugars, which are completely absorbed by the human body [27]. As chitosan can form films easily, it is used to coat and functionalize the surface of polymeric microspheres [28]. In this study, the fabrication of chitosan coated PLGA microspheres was confirmed by the FTIR spectra. Hydrophilic layers of low molecular weight chitosan (LMWC) on the surface of PLGA have been found beneficial to reduce opsonization and phagocytic uptake [18]. Therefore, LMWC is useful for both prolonged circulation and evasion of immune clearance.

Magnesium plays crucial roles in the human body. Every single cell demands adequate amount of magnesium to functionalize cellular activities. It is estimated that 2.5% to 15% of the world population suffer from some level of Mg deficiency [29]. Hypermagnesemia is a common occurrence in conventional intravenous and intramuscular magnesium delivery systems [8,9]. Therefore, controlled release of magnesium ions is a desirable characteristic for treatment of Mg deficiency. Magnesium encapsulation in a slowly degrading PLGA based polymer matrix provides the opportunity for controlled release of magnesium ions. In this study, over period of 7 days, 52.5% and 72.4% of magnesium ions were released from chitosan coated and uncoated PLGA microspheres, respectively. It was obvious that the release of magnesium ions can be prolonged using chitosan. After 7 days, less than 50% chitosan was degraded and chitosan decreased 20% release of magnesium from PLGA polymer matrix. However, the release profiles contained two expulsion processes: the first being burst release and the second, relatively constant release after the initial burst. Release from biodegradable microspheres is dependent

both on diffusion through polymer matrix and polymer degradation [30]. The initial burst effect is a result from diffusion through polymer matrix.

Cytotoxicity results demonstrated that PC12 cells and ASCs encounter acceptably low levels of damage while in contact with PLGA/chitosan, PLGA/MgG and PLGA/chitosan/MgG microspheres. Live/dead staining assay results also support the cytotoxicity of microspheres with ASCs. These results indicate favorable biocompatibility for the microspheres as a potential device for drug delivery. As magnesium uptake by cells is slow, the convenience of controlled release of magnesium could be useful for enhancing cellular uptake. This study also demonstrated a potential approach to eliminate the need of repetitive administration of magnesium as well as increase patient comfort and compliance.

5. Conclusions

In this study, chitosan coated PLGA microspheres were fabricated and investigated for controlled release of magnesium ions towards potential use in treatment of Mg deficiency. Chitosan coated PLGA microspheres showed significant control on the release of magnesium ions compared to uncoated PLGA microspheres. MgG contained PLGA/chitosan microspheres may be administered in human body by intravenous infusion or intramuscular injection because of reasonable microsphere size, biodegradability, biocompatibility and sustained magnesium ions release characteristics. However, the utility of MgG contained PLGA/chitosan microspheres remains to be explored and improved through *in vivo* studies.

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Graphical Abstract