# Pluronic and Pluronic-polysaccharide Blends for Sustained Drug Delivery and Osteogenic Efficiency

**Rangabhatla S. L. Aparna**

**A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences**

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**Thesis Title** Pluronic and Pluronic-polysaccharide Blends for Sustained Drug Delivery and Osteogenic Efficiency

**Author** Mrs. Rangabhatla S. L. Aparna

**Major Program** Pharmaceutical Sciences

**Major Advisor**

……..

(Prof. Dr. Vimon Tantishaiyakul)

**Examining Committee:**

…….….Chairperson

(Prof. Dr. Damrongsak Faroongsang)

……..……..Committee

(Prof. Dr. Vimon Tantishaiyakul)

……Committee

(Asst. Prof. Dr. Namon Hirun)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Pharmaceutical Sciences.

…….……..

(Prof. Dr. Damrongsak Faroongsang)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…..……..……..Signature
(Prof. Dr. Vimon Tantishaiyakul)
Major Advisor

…..……..……..Signature
(Mrs. Rangabhatla S. L. Aparna)
Candidate

I hereby certify that this work has not been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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(Mrs. Rangabhatla S. L. Aparna)

Candidate

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| **Author** | Mrs. Rangabhatla S. L. Aparna |
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## Abstract

Hydrogels represent a strategic role in controlled and /or sustained drug delivery. This polymeric system consists of three dimensional structures that can absorb large amount of water or biological fluids and thus can be modified for stimuli response release. In the present work novel hydrogels were prepared by combining various concentrations of 12, 14, 16, 18 and 20% (w/w) pluronic F127 (PF) with 4%(w/w) Methylcellulose (MC) to form injectable implant drug delivery systems. These blends formed gels at significantly lower concentrations of PF when compared to using PF alone. Furthermore, the gels exhibited cytocompatibility to both mouse preosteoblasts (MC3T3-E1) and mouse myoblast (C2C12) cell lines whereas the gels of 16 PF, 18 PF and 20 PF were cytotoxic to the cells. Etidronate sodium (EDS) was loaded at a concentration of $4 \times 10^{-3}$ M into these blends for yielding an osteogenesis effect. EDS loaded PF/MC blends exhibited cytocompatibility to both the osteoblast (MC3T3-E1) and myoblast (C2C12) cell lines. The EDS loaded gels further exhibited significantly greater alkaline phosphatase (ALP) activities compared to the pure gels. The ALP activity was found to be greater with increasing time. These EDS loaded gels increased proliferation of both cell lines thus indicating a bone regeneration effect. I*n vitro* release of EDS from PF/MC blends found to be extended for more than 28 days. The *in vitro* degradation test, showed that MC extensively improved the gel strength of the PF and delayed the degradation of the gels thus making them more functional for a sustained drug delivery for osteogenesis. With the aim of enhancing the sustainability of etidronate in these gel systems a simple and inexpensive approach for preparation of drug loaded nanoparticles by nanoprecipitation has been employed in the present work. Bisphosphonates are drugs used for treatment of bone disorders like Paget's disease and osteoporosis. These bisphosphonates have a very short half-life in circulation

(0.5-2 hr) for which controlled release of bisphosphonates is essential for long term therapeutic efficacy. In this work Etidronate, a bisphosphonate, was selected as a model drug. Gelatin-etidronate nanoparticles were prepared by nanoprecipitation method. These nanoparticles were characterized by Zetasizer and TEM for their size and morphology, respectively. The prepared nanoparticles were in the size range of 100-260 nm. The zeta potential value was found to be +5.86mV. The drug loading efficiency was found to be 40%. *In vitro* drug release studies revealed control release of the drug from the polymer matrix. Further these nanoparticles exhibited dose dependent cytotoxicity on mouse C2C12 cells.

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Mrs. Rangabhatla S. L. Aparna

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| | |
|---|---|
| º | Degree |
| ºC | degree Celsius |
| µg | Microgram |
| µg/ml | microgram/milliliter |
| UV-Vis | UV Visible Spectrophotometer |
| FTIR | Fourier transform infrared spectroscopy |
| nm | Nanometer |
| CM | Culture media |
| NPs | Nanoparticles |
| g/cm | gram/centimeter |
| % | Percentage |
| cm | Centimeter |
| PDI | Polydispersity index |
| TEM | Transmission electron microscopy |
| PF | Pluronic F127 |
| MC | Methyl cellulose |
| TSX | Tamarind seed xyloglucan |

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# INTRODUCTION

In the recent years over the past 30 years scientists are paying more interest in development of sustained drug delivery and controlled drug delivery systems. Among these systems the extensive focus has been laid in crafting of polymeric systems for drug delivery applications. In recent times, temperature responsive gel systems has encountered considerable interest in drug delivery. Statistics say that there is enhancement of these gel forming systems has been explored and their use in various biomedical applications including drug delivery have been reported with many patents included. This is due to the merits obtainable by *in situ* polymeric delivery systems which include administrating effortlessly, non-invasiveness, and reduction in recurrence of administration, better patient compliance and ease**.** *In situ* polymeric gel systems present an attractive substitute for attaining systemic drug effects for parental routes, which can be problematic or oral route, which may lead to low bioavailability and which may cause first-pass metabolism, in particular of proteins and peptides. Improved delivery technologies of therapeutic agents by controlling the rate at which they enter the blood stream, besides developing new drugs or bioactive compounds is the key and major focus area in improving the drug delivery systems in recent studies. [1]. In this regard hydrogels forms the basis for sustained or controlled delivery. These polymers which have a three dimensional structure and which can swell to a larger extent with large amount of biological fluids or water and which can be solid at room temperature and can gel at physiological temperature has been a part of controlled or sustained drug delivery systems[1,2].

Smart polymers can be defined as the polymers that react to small changes in surroundings such as pH or temperature, and also can overcome remarkable property changes and they can also be termed as stimuli sensitive polymers. These systems gathered numerous applications in drug delivery and tissue engineering [3]. Thermo responsive polymers are those which have a fine balance between their hydrophilic and hydrophobic groups in their structure, and their chains tend to collapse or expand

upon small change in temperature around the critical temperature and make new adjustments between the polymeric chains and the aqueous medium [2]. Some of the examples of sol-gel reversible hydrogels include Pluronics [Poloxamers or PEO-PPO-PEO triblock polymers are more recently used PEO-biodegradable polyester (PLGA, PLLA, PCL) systems. Some of these are available commercially for their application in modestly invasive injectable solutions (BST-Gel® of BioSyntech and ReGel® of Macromed). Block or graft copolymers exhibit thermoreversibility based on micellization or micelle aggregation [4,5] as shown in Figure.1.

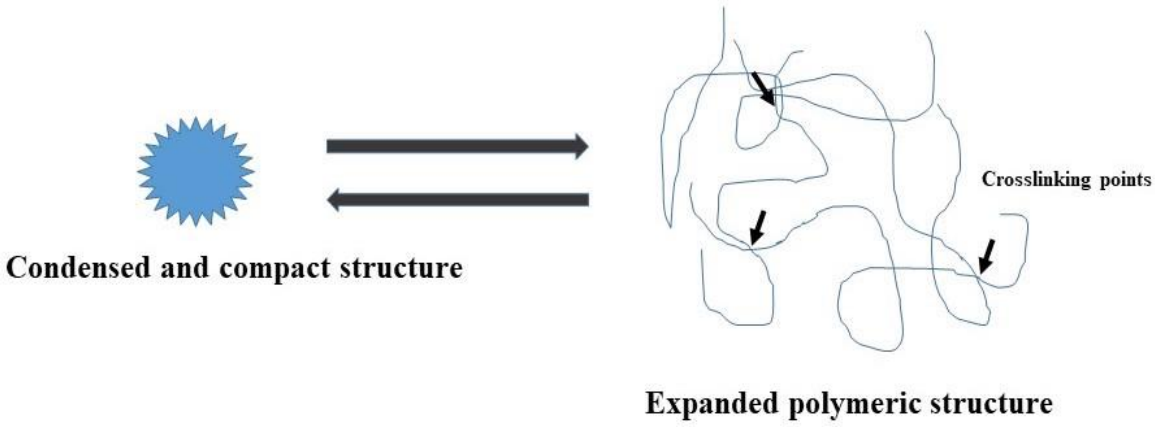


**Figure 1**: Sol-gel transition of thermoresponsive polymers [4].

Pluronics® have vast number of applications and some of these pluronics which can be used as thermoreversible gels are approved by the FDA and EPA for their use as food additives ,agricultural products and pharmaceutical excipient for varoius applications in drug delivery systems and injectable systems for tissue engineering [6,7]. polymer composition and solution concentration plays an important role in determining the gelation temperature. For instance, PluronicF127 (generic name, polaxamer 407) is a

solution at room temperature and can gel at 37 $^{0}$ C but at a concentration of 20 wt% of the polymer. They have extensive application in wound healing, burn healing etc [7].

Pluronic F127 for the effective treatment of otitis media caused by methicillin resistant *Staphylococcus aureus* (MRSA) has been used for the preparation of formulation for the local delivery of vancomycin by Lee et al. Pluronic F127 has an excellent phase transition property, which is liquid at 25 $^{0}$ C and becomes solid at physiological temperature that makes it suitable for local drug delivery [8]. More recent it has been used by mixing with isolated chondrocytes and applied on an osseous surface with a brush that formed a sticky gel in a very short time (minutes). It was observed that it could form a new cartilage at the bone cartilage interface on the osseous substrate [9]. Pluronic F127 with isolated chondrocytes for testing the formation of tissue after subcutaneous injections in mice was also applied as an injectable cartilage formulation. New cartilage formation has been demonstrated upon histological examination of all samples representing that the polymer-cell suspension is suitable and promising for orthopaedic and reconstructive surgery [9].

The ability of the Pluronics® or the transition temperature at which it can gel (physiological temperature) made them very suitable systems as injectable drug delivery carriers that can form an *in situ* drug depot. Pluronic is extensively and most commonly used application such as in sustained delivery of protein and peptides which includes insulin, growth factors ,urease, BMP and was also used as an injectable cartilage formulation[10].

Pluronic F127 also is also applied widely in cell encapsulation applications either by itself or to support cell seeding and attachment in tissue scaffolds because of its thermoreversible gelation in water . Aqueous Pluronic F127 solutions gel at physiological temperatures at concentrations of 15–20% (w/w),. The viability and proliferation of HepG2 (Human carcinoma cell) was determined for both liquid and gel formulations to check the efficiency of pluronic F127 . MTT and LDH assays were performed to determine the cell concentration and viability over a 5-day period along with the tryptan blue assay via hemocytometry and results were confirmed. At lower concentration until

5% (w/w) Pluronic F127 (liquid),high viability and cell proliferation is observed for over 5 days. However, at higher concentration of Pluronic i.e at 10% (w/w) , there was a significant decline in cell viability and there is no cell proliferation noticed. The cell encapsulation in HepG2 cells in at concentrations of Pluronic F127 ranging from 15 to 20% (w/w) (gel) resulted in complete cell death within 5 days. This holds true for the endothelial (HMEC-1) and muscle (L6) cell lines that are evaluated. Cell survival or proliferation is not affected by Cell-seeding density. To improve cell viability of these pluronics membrane-stabilizing agents such as hydrocortisone, glucose, and glycerol were added [11]. The steroid hydrocortisone exhibited marked improvement in viability, to more than 70% (with 60 nM hydrocortisone added) as compared to Pluronic alone (2%). Based on these results it can be proposed that F127 formulations complimented with membrane-stabilizing agents can serve as viable cell encapsulation materials. Furthermore, promotion of cell viability with hydrocortisone may be generally useful wide range of encapsulation materials [11].

Temperature-sensitive block copolymers bearing polyethylene oxide( PEO), are capable of mimicking the extracellular matrix at the same time enabling the formulation of aqueous systems easily syringable at room temperature that can lead to highly viscoelastic gels that gel at 37 ºC[12,13]. One more major advantage is the low surface tension of the gels that allows the loading of proteins without denaturing in the aqueous phase of the hydrogel, while the transition from sol-to-gel makes the sustained release possible under physiological conditions [14,15]. Very few studies are carried out with polypropylene fumarate-PEG[16] and poly(ethylene oxide)-poly(propylene oxide)-poly(ethyleneoxide) for tissue engineering applications, However pluronic *in situ* gelling systems facilitated to regulate the delivery of BMP-2 and BMP-7 [17] when compared to other biodegradable polymers, such as PLA or PLGA, which may not be completely reabsorbed and sometimes they appear as voids or black holes in the formed bone when verified radiographically .In addition, Pluronic can be completely restored by the new bone [18]. Except for minor accumulation in the urine, brain, lung and liver, pluronics are rapidly excreted in the urine, and there is no risk of reproductive and/or developmental toxicity [18,19].

Pluronic F127 has been studied extensively as an in-situ drug delivery system in delivery of many drugs. However, very few papers reported the cytotoxicity of Pluronic F127. Pluronic F127 at a concentration above 10 % is found to be cytotoxic on, endothelial (HMEC-1) and muscle (L6) cell lines [11]. But for Pluronic F127 to form thermoreversable systems, the concentrations should be above 16% which render the gels cytotoxic. Taking this problem into concern in the present work we wish to reduce the cytotoxicity of in-situ gelling systems by combining these pluronics with polysaccharides to form conjugated systems.

Owing to the biodegradable nature of the polysaccharides along with their thermo reversible ability they have been extensively studied for the development of hydrogel systems which can be used both for sustained drug delivery and tissue engineering.

A water-soluble polymer and cellulose polysaccharide derivative, methyl cellulose is widely used as a pharmaceuticals excipient as binder or thickener, foods, ceramics, etc. MC usually experiences a two-stage thermoreversible gelation in aqueous solution above a critical micellar temperature. Results suggest that different salts have different effect on gelation temperature. However, for pure Methyl cellulose solution, gelation temperature is always occur in the region of ∼50–70 ◦C[20], and therefore it cannot be used as an injectable product for gelation in vivo at 37 $^{0}$C. So instead of modifying the polymer by chemical crosslinking or grafting or by using harsh solvents they can be blended with other polymers .Blending is an vital method for modifying and/or improving the physical properties of polymeric materials, and thus escalating the range of their application. Further various blends are used in tissue engineering applications [20,25].

In order to enhance the osteogenic efficiency of these thermoreversable gels, they can be loaded with bisphosphonates. Bisphosphonates are chemically analogs of pyrophosphates that has a non hydrolysable P-C-P backbone with two side chains $R^1$ and $R^2$.The two phosphonate groups assist in both binding to bone mineral and for its anti resorptive activity. Bisphosphonates are majorly classified into two types based on the groups present on the side chains. The mechanism of action of bisphophonates is briefly

described as in Figure.2.The presence of hydroxyl groups or amino group in the $R^1$ chain improves the binding to calcium minerals. Similarly the presence of nitrogen or amino group on the $R^2$ side chain affects the binding to hydroxyapatite and also enhances the anti resorptive property[21]. Based on this the bisphosphonates are classified as Nitrogen containing bisphosphonates which act by inhibiting (Farensyl diphosphate synthase) in melovonate pathway eg palmidronate, alendronate, ibandronate etc) and non—nitrogen containing bisphosphonates which act by incorporation into ATP(eg :clodronate, tiludronate and etidronate). Though nitrogen containing bisphosphonates are the first choice of drugs for bone resorption, it was reported that these bisphosphonates cause necrosis at injection site whereas etidronate is cytoprotective[22].

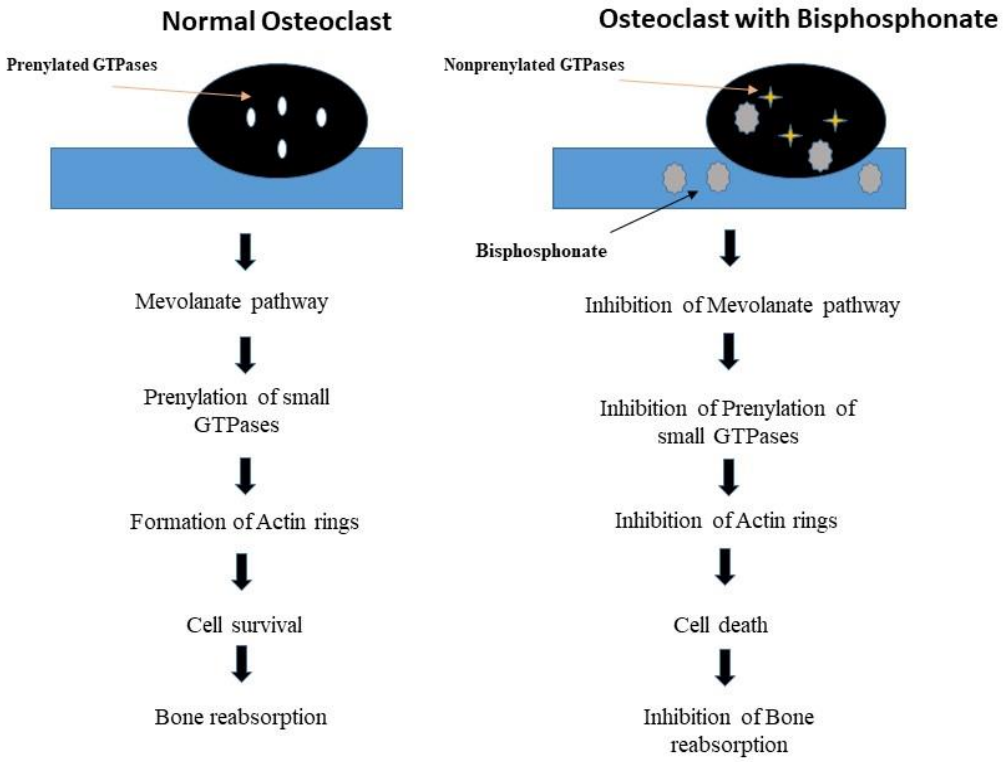


**Figure 2**: Bisphosphonates mechanism of action [24]

Bisphosphonates have long history in mitigating bone regeneration. Srisubut et al were successful in regenerating bone by incorporating Alendronate in bioactive glasses [23]. Similarly, Gao et al used zoledrinoic acid in combination with fibroblast growth factor for bone regeneration in osteoporosis model. In addition to the above mentioned uses in bone regeneration bisphosphonates can also be used in various conditions like Paget disease, breast cancer and prostate cancer [24].

However, bisphosphonates have few disadvantages like poor bioavailability (less than 2% oral absorption). Owing to this nature of these drugs other routes of administrations like intravenous, subcutaneous and intramuscular were investigated. Even intravenous (IV) route also suffers major drawbacks like higher excretion rates which cause payload in the kidneys. Thus local delivery of bisphosphonates came into existence which could deliver the treatment to the site with minimal systemic side effects. Liposomes, nanoparticles and microparticles were explored for local delivery of these drugs.

Intra articular delivery of bisphosphonates was achieved by nanoparticle liposomes enhance their potency by a factor of 20–200 [25]. Chitosan microspheres of different composition as drug carriers have also been explored for injectable delivery systems [26]. Extended release of clodronate was achieved by incorporating drug in biodegradable polylactide based microspheres which showed in vitro release of up to 70 days [27,28]. Similarly, Sol–gel derived silica microspheres have also been tested for this drug delivery systems [29,30]. Most of the bisphosphonates were formulated either in nano/microparticle forms along with silica based drug delivery systems because of their ability to be degraded by innate osteoblasts. Many stratagies were explored for these types of delivery systems like sol-gel method, adsorption of drug on to silica particles, incorporation into mesoporous silica etc. [30]. All the methods for drug-incorporation have their own advantages, and the right method should be chosen based on the end use. However, these delivery systems present a rapid release of drug due to mesoporous nature of the silica and controlled release of drug is essential for induction of osteogenesis. In our study we investigated the feasibility of formation of nanoparticles of

etidronate with gelatin by nanoprecipitation method and evaluated the osteogenic effect of these nanoparticles.

Similarly, injectable gels occupy a vital role in delivering osteogenic molecules. Injectable scaffolds offer advantages over solid scaffolds because of their fluidic nature, non-invasive method. These gels can be loaded with various drugs and growth factors to promote osteogenesis. Owing to all these reasons injectable gels provide a wide platform for delivering drugs. Chitosan- Alginate gels loaded with cells and BMP-2 were fabricated for inducing osteogenesis. gelatin based biocompatible nanoparticles will provide an alternative for conventional treatment making a sustained release of the drug[31].

Polymeric nanoparticles (NPs) have provided several advantages which may involve relatively high intestinal uptake probably due to particle size and charge properties. Numerous advantages of gelatin such as availability of functional groups that are easily accessible, low antigenicity, and which can offer many opportunities for pairing with crosslinkers and targeted ligands, make it a desirable biomaterial [32]. Furthermore gelatin is widely used as in parentral formulations and also stabilizer in vaccines. In this study, a simple, rapid instantaneous method is used for preparation of NPs from different polymers as it does not require any kind of comprehensive treatment such as, usage of harsh chemicals/solvents, sonication and high temperature. Gelatin type A is basic in nature with many amino groups which will entrap etidronate which is negatively charged to give a complexation product. Type of gelatin is chosen based on the entrapping drug molecule. The aim of the present work is to perform preliminary studies on cytotoxicity and release properties of prepared NPs.

## OBJECTIVES

1. To prepare and characterize etidronate loaded methyl cellulose- pluronic F127 thermoreversable gels.
2. To investigate the osteogenic potential of etidronate methyl cellulose- pluronic F127 thermoreversable gels
3. To prepare and characterize Etidronate gelatin nanoparticles and to check their cytocompatibility

## Significant Results & Discussion

### Preparation of pluronic/methyl cellulose (PF/MC) samples

Different concentrations of PF solutions ranging from 16, 18 and 20% w/w were prepared by following Lin et al., 2004. 4% MC solutions were prepared by dispersing MC in purified water with constant mixing at cold condition. Required amount of PF was then added in the MC solution to get concentrations ranging from 12 to 20% w/w of PF in MC (4% w/w) and these blends were labeled as 12PF/MC to 20PF/MC, respectively. The final blends were thoroughly dissolved and kept at 8ºC ensuring complete solubilization. For cytotoxicity testing PF and MC powder samples were UV sterilized and dissolved in sterile water under aseptic condition. [33]. 0.2 g of corresponding PF/MC gels or PF were added to a 24-well plate and allowed for gelation followed by addition of PBS (1 mL) in each well. After incubating the plates for 1 day the extracts of PBS were sterilized by filteration. The filtered solutions (100 µl) were further studied for the cytotoxicity [33]. Etidronate sodium (EDS) at concentrations ranging from $10^{-4}$ to $10^{-5}$M has been previously found biocompatible to various cell lines [34].

### Viscosity of the samples

The rheologies of the gels were measured by Malvern Instruments, Gemini HR-nano, UK with geometry of cone plate at (diameter 4cm, angle1). Silicone oil is used to prevent evaporation of these samples from the corners. The flow curves of different gel combinations of gels (20 %PF alone and highest and lowest concentration of blends with EDS) at room temperature are shown in Figure.3. As the concentrations of PF increased in the blends the viscosity also increased. 20PF/MC/EDS gels showed higher viscosity than that of 20PF/EDS clearly indicating the role of PF. Thus MC and PF play a vital role in influencing the viscosities of the systems displaying pseudo plastic behavior. These behaviors could help in easy flow of the liquid through the syringe which is required for parenteral use.

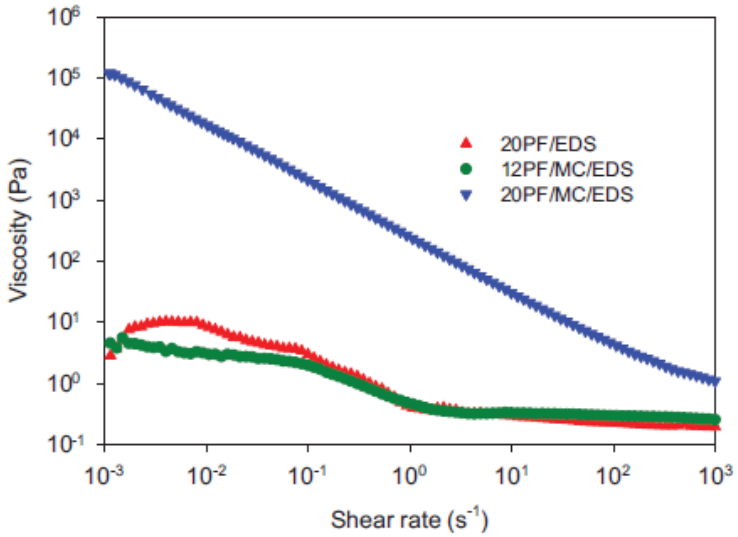


**Figure 3**: Shear rate-viscosity flow curves for samples containing various concentrations of PF, 4% w/w MC and 4 x10 $^{-3}$M EDS (EDS) at 25ºC.

**Evaluation of cytotoxicity**

C2C12 and MC3T3-E1 cell lines were used to determine cytotoxicity [35]. The cells at a density of 2 x10 $^{4}$ cells per well were plated in 96-well plates. The cells were incubated in DMEM at 37ºC, 5% $CO_2$ for 24 h. 100 µL of the extract solutions from different blend were inoculated and incubated in DMEM at 37 ºC, 5% in an $CO_2$ incubator for 1 day. Zinc acetate at a concentration of 100µg/ml was used as positive control and the medium for cell culture was used as negative control respectively [36]. After incubation media was removed and washed with 100µL of PBS followed by addition of MTT reagent 100 µL Cells were incubated for 4 h at 37 ºC [37] followed by addition of DMSO and the absorbance was measured at 570 nm using a Beckmans coulter microplate reader, CA, USA. Similarly cytotoxicity of EDS was also determined. All the gel samples of blends were also evaluated for cytotoxicity. There is a considerable difference in the cell viability of the PF and PF/MC on MC3T3-E1 cell (Figure.4). 16PF and 18PF gels showed cell viability of 65%. 20PF gels were found to be highly toxic with cell viability of 61.1%. In contrast PF/MC blends showed excellent biocompatibility on MC3T3-E1 cells. The 12 and 14PF/MC blends showed superior cell viability than the

media alone ($p < 0.5$). Similar kind of results were obtained on C2C12 cells. Presence of MC as a membrane stabilizing agent could enhance the biocompatibility of pluronic.[40,41]

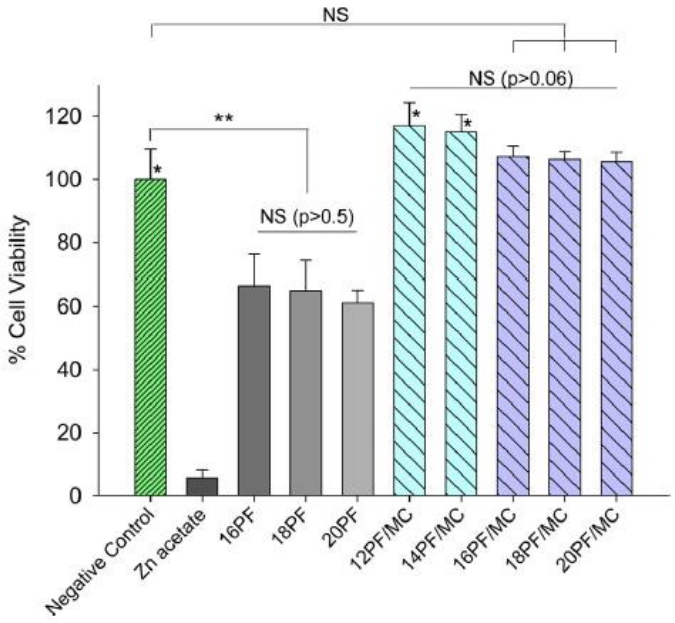


**Figure 4**: Cytotoxicity of various concentrations of PF and the blends of PF and 4% (w/w) MC on MC3T3-E1 cells. Cytotoxicity of positive (cell culture medium) and negative (100 ppm zinc acetate) controls are also shown. Data are shown as mean SD, n = 8 per group; *$p < 0.05$, **$p < 0.001$.

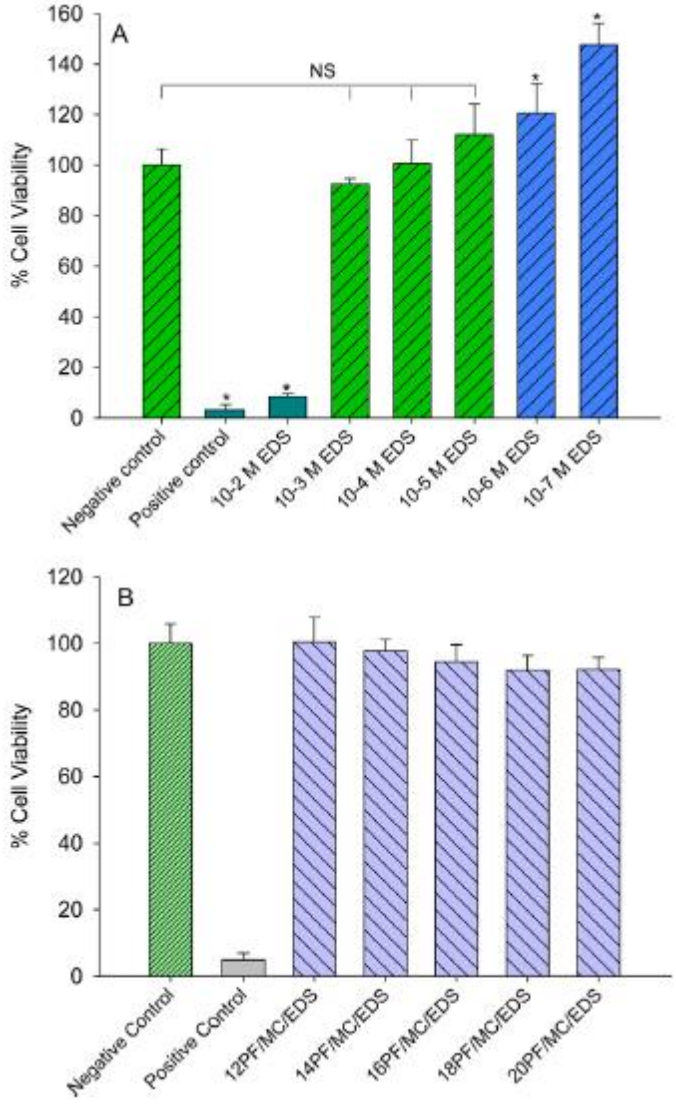


**Figure 5:** Cytotoxicity of (A) various concentrations of EDS and (B) the blends of 4 x10 $^{-3}$M EDS, 4% (w/w) MC and various concentrations of PF on MC3T3-E1 cells. Cytotoxicity of positive (cell culture medium) and negative (100 ppm zinc acetate) controls are also shown. Data are shown as mean SD, n = 8 per group; *p < 0.001.

In this study 3 and 4% w/w MC blends are studied. Further, the different formulations of PF with 4% w/w MC showed a greater cytocompatibility than the control which lead us to choose 4% w/w MC for other studies in this work . EDS concentrations at less than $10^{-3}$M were found to be non-toxic to both cells. In order to prove the same concept etidronate at concentrations ranging from $10^{-2}$ M to $10^{-7}$ M has been evaluated for cyctotoxicity Figure.5A.

1 mg/mL ($4 \times 10^{-3}$ M) concentration of EDS has showed biocompatibility on MC3T3-E1 cells along with PF/MC blends. When the 1 mg/ml of EDS was loaded in these gels, the cell viability of the osteoblast cells were similar to negative control. But the lower concentrations of PF showed better compatibility than high PF concentrations but at the same time statistically not different as shown in Figure.5B

Osteogenic differentiation of the prepared gels

The ALP activity was measured as a marker of osteogenic differentiation using para-nitrophenyl phosphate (pNPP) method [38]. The ALP activity was determined at different time points starting from 1, 3, 5 and 7 days. Cells grown on the gels were lysed at 4ºC using required quantity of Triton X for a period of 30 min. The so obtained cell suspension was centrifuged at 12000 *g* at 4ºC for 15 min. 100 µL of the supernatant was moved to a new well plate followed by the addition of 500 µL of substrate mixture (20 mM pNPP). The total mixture was incubated at 37ºC for 30 min. NaOH was added to stop the reaction and the absorbance was measured using a micro plate reader at given wavelength. Osteogenic efficiency of all PF/MC and PF/MC/EDS were shown in Figure. 6A & 6B. There is profound enhancement of ALP activity on MC3T3- E1 cells in presence of gels from day 1 to day 7. Higher the concentration of PF resulted in higher ALP activity. 20PF/MC blend with maximum PF concentration showed notable increase in ALP activity compared to the control ($p < 0.001$).

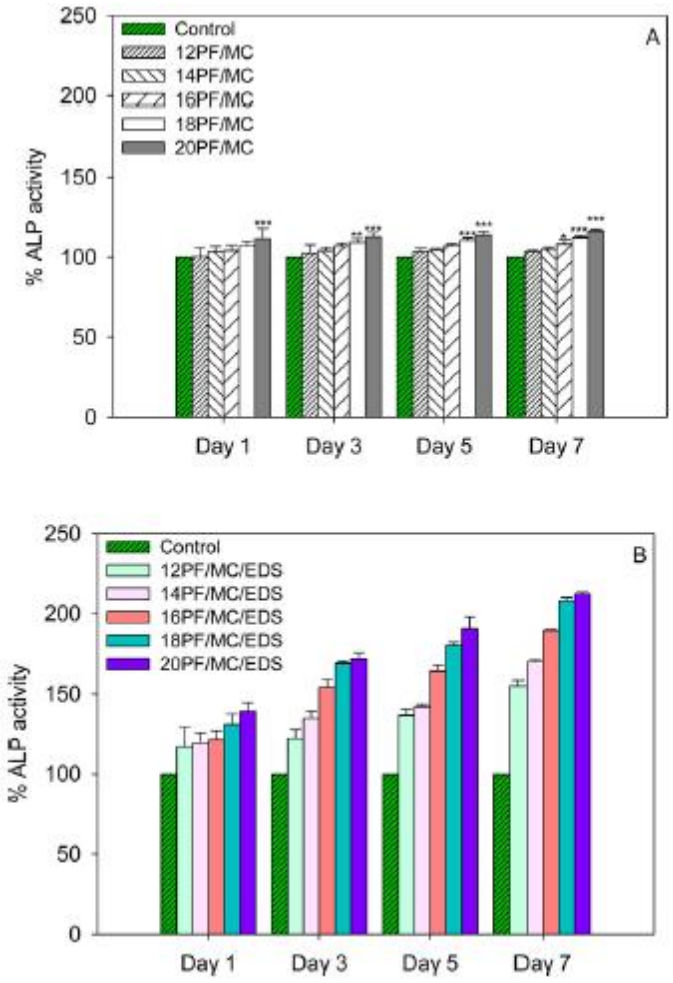


**Figure 6**: Time course of changes in the ALP activity of various concentrations of PF (A) in 4% (w/w) MC and (B) in 4% (w/w) MC and 4 x $10^{-3}$ M EDS on MC3T3-E1 cell line (mean SD, n = 4 per group; compare to the control (A) *p < 0.05, **p < 0.01, ***p < 0.001, (B) p < 0.001 for all PF/MC/EDS blends).

In vitro drug release

Membrane less method was used to monitor the release of EDS (1 mg/mL) from the gels [33]. . Liquid preparation of PF alone and PF/MC blends with etidronate were transferred to flat-bottomed vial having internal diameter of 18 mm and allowed it to form a gel and incubating it at 37ºC for 5 min. On top of the gel 8 mL of 0.9% NaCl w/w solution was placed gently. 3ml of samples were collected at preset time points and

restored with same amount of NaCl [39]. Collected samples were Cu-complexation with CuSO4 in acidic pH (2.8) using $HNO_3$ and analyzed by UV Spectrophotometer Agilent Technologies, USA at a wavelength of 240 nm [40,41]. PF gels showed slow release of hydrophilic drugs such as vancomycin and mitomycin C [42]. In present study similar release pattern was observed for EDS which is hydrophilic. Release profiles of EDS from 20PF and all the combinations of PF/MC are shown in Figure.7. It was found that there is an early burst of release from the gels followed by a prolonged release during later days. Pluronic alone at concentration of 20% (20PF) could sustain the drug release for 5 days only. But blends of PF/MC could prolong the release and could hold the drug for 28 days. This could be due to entrapment of EDS on the surface of micelles [42]. Dense internal structure of high concentration of PF, presence of MC and viscosity of the blends played active role in retarding the release of EDS from the blends.

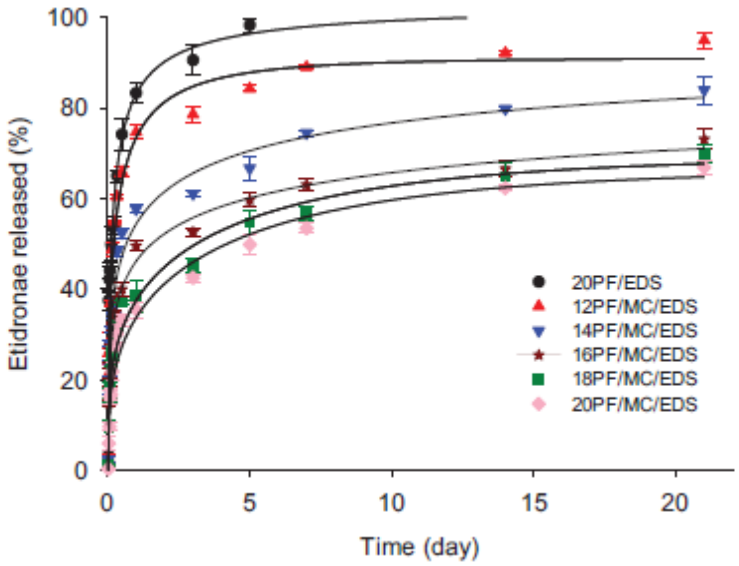


**Figure 7**: In vitro release of EDS from the gels of 20PF and the blends of various concentrations of PF and 4% (w/w) MC and 4 x $10^{-3}$M EDS at 37ºC (mean ±SD n=3)

**Preparation of gelatin-etidronate nanoparticles**

Preparation and characterization of nanoparticles:

Nanoparticles (NPs) were prepared by Nano precipitation method which was described elsewhere [32]. 5 mg of etidronate disodium was dissolved in 2 mL of distilled water followed by addition of 40 mg of gelatin with continuous stirring for 30 min at 50 °C and solution was labeled as solution A. This solution A is then added drop to drop to 30 mL of ethanol containing 1.28 g of pluronic F127. Final pluronic F127 to gelatin mass was maintained at 32:1. The so obtained colloidal solution was then centrifuged and freeze dried. Similarly, blank gelatin NPs were also prepared by same procedure without the drug. The freeze dried samples were weighed and the yield of NPs obtained was calculated . Blank particles are calculated similarly. The drug loading efficiency was determined using Equation.1. The zeta potential, size distribution and poly dispersity index (PDI) was measured using zetasizer (Zetasizer Nano ZS90, Malvern, UK). Furthermore, the morphology of particles was analyzed by Transmission Electron Microscopy (TEM JEOL-2010). The average size of blank NPs was 107.2 nm and that of drug loaded gelatin NPs was 262 nm. The mean size of drug loaded gelatin NPs was slightly higher than that of blank gelatin NPs . Particle size of the NPs is dependent on the concentration of ethanol;Higher the concentration of ethanol, the lower the particle size. In our experiments we have used 90% ethanol in order to achieve minimum solubility of gelatin and etidronate. The zeta potential

values and PDI values were given in Table.1. Generally, the lower PDI values indicated the homogeneity of the dispersion.

**Table 1**. Mean size, zeta potential and poly dispersity index of gelatin and gelatin-etidronate nano particles.

| Parameter | Gelatin nanoparticles | Drug loaded gelatin nanoparticles |
|---|---|---|
| Mean particle size | 107.2 ± 3.27 (nm) | 262 ± 5.64 (nm) |
| Zeta potential | + 5.83 mV | + 1.98 mV |
| Polydispersity index (PDI) | 0.231± 0.005 | 0.509 ± 0.005 |

Because of high ethanol concentration used in this study, the PDI is slightly high . Zeta potential values of blank and drug loaded nanoparticles differed because of interaction between positively charged gelatin and negatively charged etidronate. Pure gelatin nanoparticles with unoccupied aminogroups showed higher positive charge than the drug loaded nanoparticles.

**Equation 1.** Entrapment efficiency of the nanoparticles

$$EE = (\text{Total amount of drug–free drug} / \text{total amount of drug}) *100$$

**Invitro release**

5 mg of NPs were diluted with 5 mL of PBS 7.4 and loaded in dialysis membrane with molecular cutoff of 14,000 to 20,000 KDa and this membrane was immersed in 50 mL of the release medium. The experiment was carried out at 37 °C at 40 rpm in a shaking incubator (Diahan Labtech Co. Ltd). Sample (2 mL) was withdrawn at regular intervals and replaced with fresh PBS (pH 7.4). The samples were analyzed by copper complexation method at 240 nm as previously described [40,41]. PBS 7.4 is shown in Figure.8. The release of etidronate from gelatin NPs was found to be in a sustained manner. The initial burst release may be attributed to the etidronate that was accumulated at the surface of NPs that could easily diffuse to the external medium . The percentage release from the NPs increased consistently and reached a plateau at about 12 h. The modified release of etidronate may be attributed to the polyelectrolyte complexation between the acid groups of etidronate and the amine groups of gelatin protein matrix .

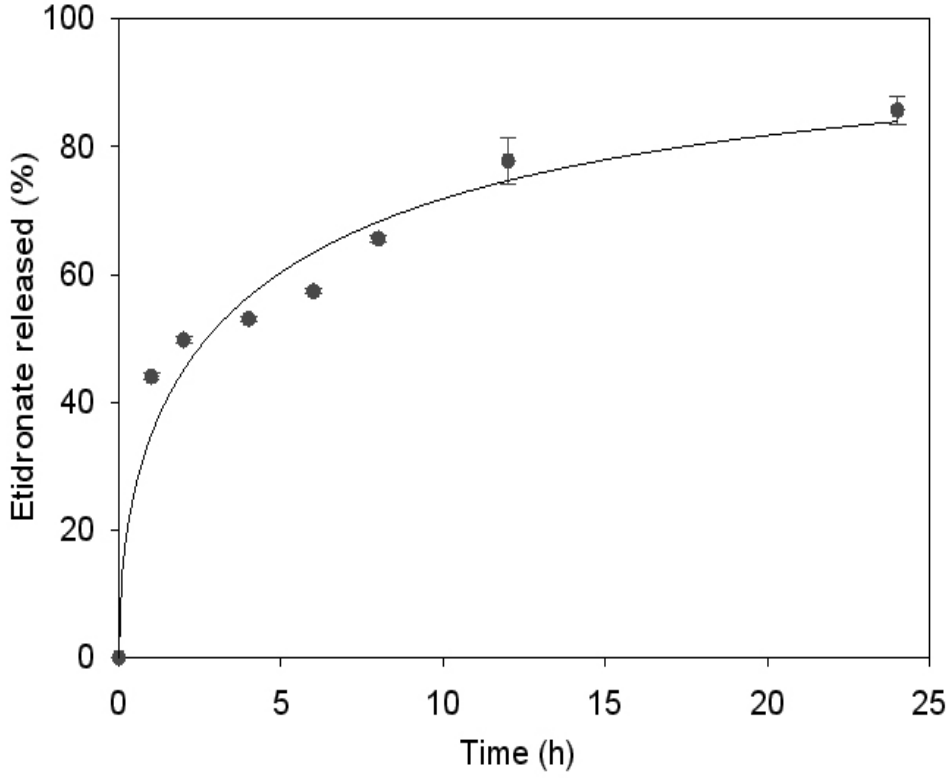


**Figure 8:** *In vitro* release of etidronate from gelatin-etidronate nanoparticles

**Biocompatibility**

Initial biocompatibility of the prepared NPs was conducted on C2C12 cell lines by MTT assay. C2C12 Cells at a density of $3 \times 10^4$ cells/well were seeded in 96-well plates and incubated at 37°C for 24 h, followed by media removal and washings with PBS. Different concentrations of NPs were inoculated ranging from 50 to 250 μg/mL. Subsequently, the cell viability was determined by measuring the absorbance at particular wavelength by using microplate reader. The biocompatibility of gelatin-etidronate NPs significantly decreased at a concentration of 200 μg/mL Figure.9. However, the NPs increased the biocompatibility of etidronate than that of free drug which exhibits cytotoxicity at a concentration less than 2.5 μg/mL (100 μM) to many cell lines.

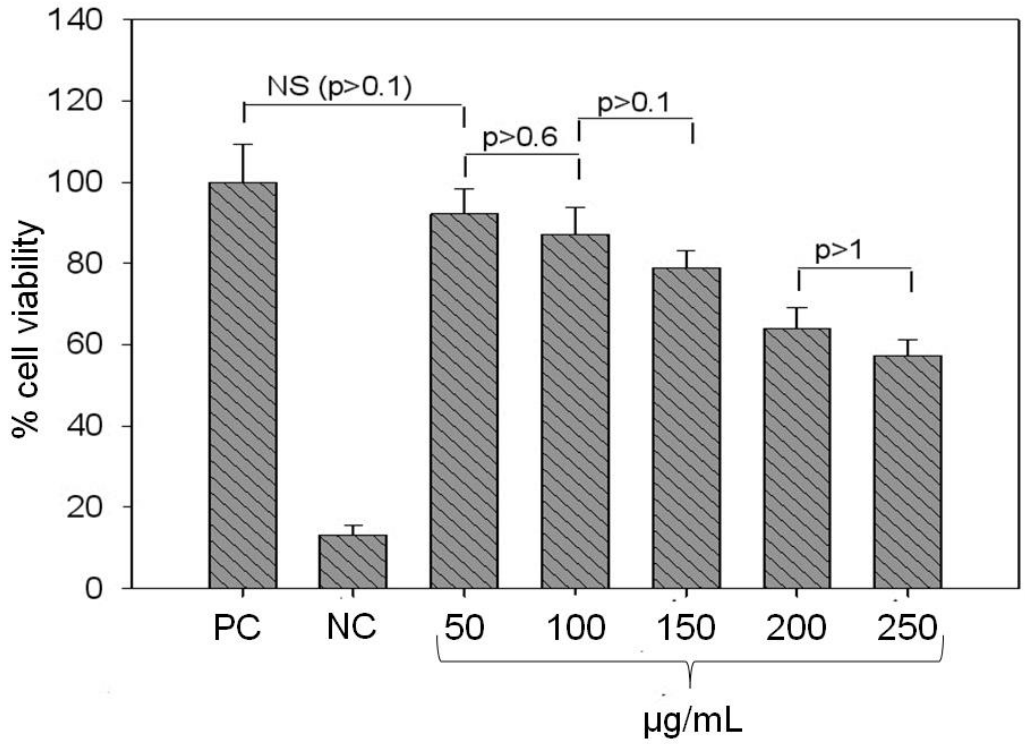


**Figure 9:** Cytotoxicity of gelatin-etidronate nanoparticles at various etidronate concentrations

## CONCLUSIONS

PF/MC gels were prepared with improved biocompatibility and enhanced stability excluding toxic chemicals and organic solvents which would suffice the needs for drug delivery and tissue engineering. PF at a concentration of about 12% could gel in presence of MC which are rendered non-cytotoxic by the presence of MC. This is supported by increase in cell viability of MC3T3-E1 and C2C12 cells in the presence PF/MC gels when compared to pure PF gels. Polymer blends of 12PF/MC, 14PF/MC showed sol state at room temperature and as a gel at 37ºC. This property can be effectively used for injecting the gels at the site and subsequent gelation for controlled release of the active substances. The Etidronate loaded gels showed increased osteogenisis of MC3T3-E1 cells and moreover they showed osteogenic differentiation of C2C12 (myoblasts) cells that usually would not express ALP. The invitro release profile of the blends PF/MC had a sustained release of EDS for 28 days. Therefore, these gels would be able to sustain the release of EDS as these gels can be injected at the site of application that would enhance its application in bone remodeling. NPs of gelatin-etidronate and gelatin nano particles were effectively prepared by nano precipitation method with satisfactory drug loading efficiency. The low PDI values (< 1) indicated homogeneity of the NPs. The NPs exhibited slow release without much burst release. The cytotoxicity of etidronate was markedly reduced in the gelatin NPs, thus enhancing the biocompatibility of etidronate. Hence gelatin-etidronate NPs are promising means for bone related disorders.

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# APPENDICES

## APPENDIX A

**Conditions for determination of Etidronate by UV –Visible spectrophotometer**

prepared samples by dissolving required amount in 1.5mmoles of copper sulphate and 1.5mmoles of nitric acid pH 2.8 and measured absorbance at 232 nm

**a. Standard curve used for Invitro release experiments**

**Table 2.** Concentration Vs Absorbance for determination of standard curve of Etidronate by UV-Visible spectrophotometer

| Concn in ppm | absorbance |
|---|---|
| 10 | 0.156 |
| 20 | 0.207 |
| 30 | 0.257 |
| 40 | 0.321 |
| 50 | 0.365 |
| 60 | 0.423 |
| 70 | 0.502 |
| 80 | 0.541 |
| 90 | 0.603 |
| 100 | 0.656 |
| 200 | 1.206 |

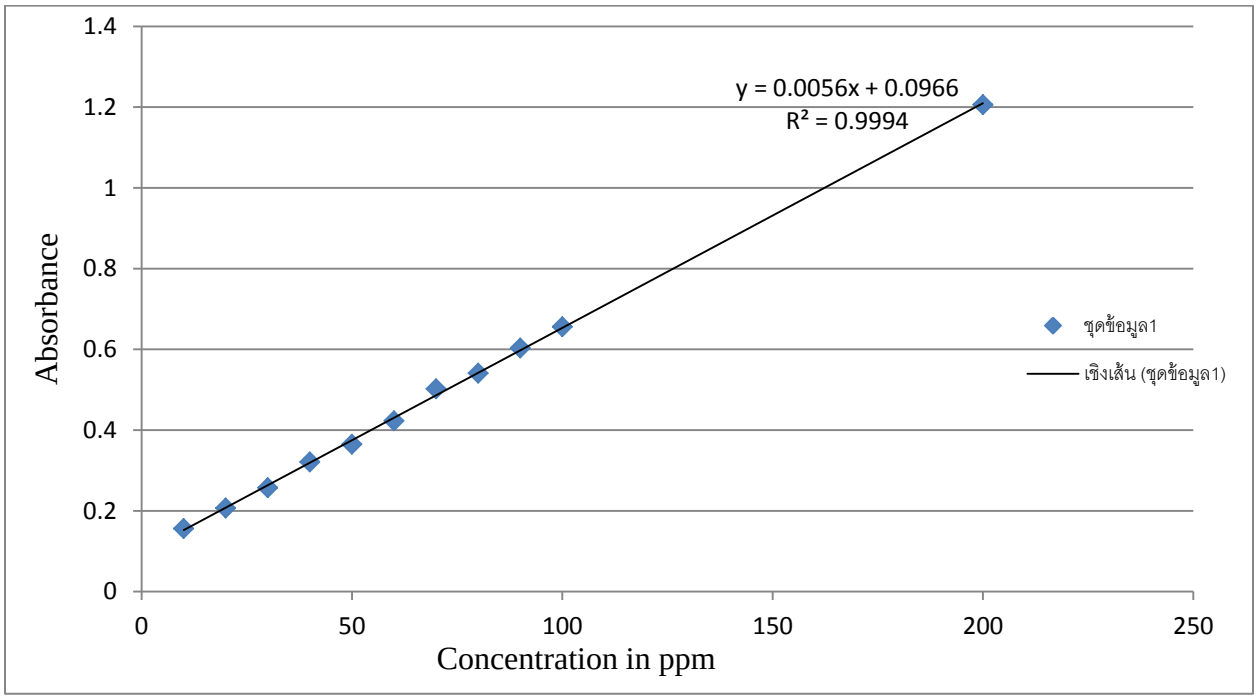


**Figure 10.** Standard Curve of Etidronate by UV Visible Spectrophotometer

# APPENDIX B

## CELL CULTURE MEDIA

**1.Alpha Minimum Essential Medium (α** MEM) Gibco®

α MEM (α Minimum Essential Medium) is widely used for mammalian cell cultures. α MEM can be used with a variety of suspension and adherent mammalian cells, including osteoblasts, keratinocytes, primary rat astrocytes, and human melanoma cells. MEM α is a modification of Minimum Essential Medium (MEM) that contains non-essential amino acids, sodium pyruvate, lipoic acid, vitamin $B_{12}$, biotin, and ascorbic acid. This product is made with Earle's salts. MEM α contains no proteins, lipids, or growth factors. Therefore, MEM α requires supplementation, commonly with 10% Fetal Bovine Serum (FBS). MEM α uses a sodium bicarbonate buffer system (2.2 g/L), and therefore requires a 5–10% $CO_2$ environment to maintain physiological pH.

**2.DMEM (Dulbecco's Modified Eagle Medium)** Gibco®

DMEM (Dulbecco's Modified Eagle Medium) is a widely used basal medium for supporting the growth of many different mammalian cells. Cells successfully cultured in DMEM include primary fibroblasts, neurons, glial cells, HUVECs, and smooth muscle cells, as well as cell lines such as HeLa, 293, Cos-7, and PC-12. DMEM is unique from other media as it contains 4 times the concentration of amino acids and vitamins than the original Eagle's Minimal Essential Medium. DMEM contains no proteins, lipids, or growth factors. Therefore, DMEM requires supplementation, commonly with 10% Fetal Bovine Serum (FBS). DMEM uses a sodium bicarbonate buffer system (3.7 g/L), and therefore requires a 5–10% $CO_2$environment to maintain physiological pH.

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# Fabrication of pluronic and methylcellulose for etidronate delivery and their application for osteogenesis

Aparna Sai Laxmi Rangabhatla[a], Vimon Tantishaiyakul[a,b,*], Kwunchit Oungbho[c], Onpreeya Boonrat[a]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90110, Thailand
[b] PSU Center of Excellence for Drug Delivery System, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90110, Thailand
[c] Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90110, Thailand

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ABSTRACT

Novel hydrogels were prepared by blending 4% (w/w) methylcellulose (MC) with various concentrations of 12, 14, 16, 18 and 20% (w/w) pluronic F127 (PF) to form injectable implant drug delivery systems. The blends formed gels using lower concentrations of PF compared to when using PF alone. Etidronate sodium (EDS) at a concentration of $4 \times 10^{-3}$ M was loaded into these blends for producing an osteogenesis effect. The pure gels or EDS loaded gels exhibited cytocompatibility to both the osteoblast (MC3T3-E1) and myoblast (C2C12) cell lines whereas the gels of 16PF, 18PF and 20PF were very cytotoxic to the cells. The EDS loaded gels demonstrated significantly greater alkaline phosphatase (ALP) activities compared to the pure gels. The longer exposure time periods of the samples to the cells, the greater was the ALP activity. These EDS loaded gels significantly increased proliferation of both cell lines thus indicating a bone regeneration effect. The PF/MC blends prolonged the *in vitro* release of EDS for more than 28 days. Based on the *in vitro* degradation test, the MC extensively improved the gel strength of the PF and delayed the degradation of the gels thus making them more functional for a sustained drug delivery for osteogenesis.



## 1. Introduction

Hydrogels are hydrophilic polymeric networks that have been widely used for pharmaceutical and biomedical applications. They have created a great deal of interest owing to their numerous advantages such as their biodegradability and cell encapsulation. Exceptionally, thermoreversible hydrogels with an ability to undergo a phase transition from a sol to a gel form following an external stimulus, such as temperature, have been used for several applications in drug delivery (Fundueanu et al., 2009; Klouda et al., 2011; Misra et al., 2009), tissue engineering (Tang et al., 2010b), biosensors (Gant et al., 2010) and myocardial injection therapy (Nelson et al., 2011). Injectable hydrogels, in particular, can be used as encapsulation devices that is they are in a sol state at room temperature but form a gel at a physiological temperature. As the material is injected in the liquid state, it can maintain a high local concentration of a drug in the surrounding tissues. One of the examples of sol–gel reversible hydrogels includes pluronics [poloxamers or poly(ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) (PEO–PPO–PEO) triblock copolymers] that exhibit thermoreversibility based on micellization or micelle aggregation (Kohori et al., 2002; Neradovic et al., 2001). The thermoreversible nature of pluronic materials permit them to be used as a carrier for oral, topical, intranasal, vaginal, ocular and parentral administration (Escobar-Chávez et al., 2006). In more recent years pluronics have been explored for applications in tissue engineering and biomedical applications. Although pluronic has been used for the *in-situ* drug delivery of various drugs, there are certain issues of concern such as possible cytotoxicity, mechanical strength as well as their stability due to its delicate network. Pluronic F127 (Escobar-Chávez et al., 2006) (PF, $PEO_{106}$–$PPO_{70}$–$PEO_{106}$) has been used in this study. PF above 10% was found to be cytotoxic towards HepG2, HMEC-1 (endothelial) and L6 (muscle) cell lines (Khattak et al., 2005). For PF to form a thermoreversible gel, its concentration should be higher than 15% (Sun and Raghavan, 2010) which causes cytotoxicity. Nevertheless this cytotoxicity of PF can be reduced by using membrane stabilizing agents such as hydrocortisone, glucose and glycerol (Khattak et al., 2005).

* Corresponding author at: Nanotec-PSU Center of Excellence for Drug Delivery System, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90110, Thailand. Fax: +66 74428239
*E-mail addresses:* vimon.t@psu.ac.th, vimon8864@yahoo.co.th (V. Tantishaiyakul).

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0378-5173/

Methylcellulose (MC) is a polymer derived from cellulose that is widely used as a drug excipient. It undergoes thermoreversible gelation in the region of 50–70 °C, hence MC alone cannot be used as an injectable material because that would require it to be in the gel form at body temperature (37 °C). A blend of MC and chitosan in the presence of salts has been reported to be useful for applications in tissue engineering (Tang et al., 2010a). Furthermore, a blend of MC and PF can form a gel at body temperature in the presence of ammonium sulfate and this blend prolonged the release of docetaxel (Kim et al., 2012). MC can protect the cells from a physical stress (Kuchler et al., 1960) and has a stimulatory effect on cells (Mizrahi and Moore, 1970). Therefore, it was of interest to blend MC and PF and to investigate the possibility that perhaps the blend was a gel at body temperature. In addition, based on the known effects of MC on the cells, MC may be able to reduce the cytotoxicity of PF.

Bisphosphonates are pyrophosphate analogues that are resistant to hydrolysis and have exceptional affinity for bone tissue. Bisphosphonates have been known to inhibit the activity of osteoclasts (Czuryszkiewicz et al., 2005). The anti-resorption activities of bisphosphonates make them the drug of choice for several bone diseases such as paget's disease, multiple myeloma, bone metastases, hypercalcemia, osteoporosis and breast cancer (Czuryszkiewicz et al., 2005; Nancollas et al., 2006). However they have poor bioavailability (oral absorption less than 2%) that has resulted in investigating other administration routes such as intravenous, subcutaneous and intramuscular therapy (Salzano et al., 2011). However intravenous (IV) administration of bisphosphonates suffers another drawback as 30–80% of drug can be excreted through the urine and this may cause renal failure due to the formation of calcium complexes in the blood (Grainger, 2012). Thus localized delivery of bisphosphonates has, in recent years, focused on the ability to enhance their therapeutic efficiencies and minimize their adverse effects. At the present time liposomes, a microsphere based delivery system, have been explored for the local delivery of bisphosphonates (Czuryszkiewicz et al., 2005). Nitrogen containing bisposphonates are many times more potent than the non-nitrogen containing bisphosphonates such as etidronate sodium (EDS). In this work, EDS was the drug of choice since the latter may cause necrosis (Tanaka et al., 2013). Moreover nitrogen containing bisphosphonates were cytotoxic to periodontal tissues.

The aim of this present study was to probe the feasibility of incorporating EDS into the gels and check the blends of PF and MC for use as injectable thermoresponsive gels for local delivery of drugs. The interactions between MC and PF were determined using attenuated total reflection Fourier transform spectroscopy (ATR-FTIR). The viscosity of the blends was determined. The morphologies of the freeze dried gels were examined by environmental scanning electron microscopy (ESEM). The *in vitro* release of EDS and the degradation of the gels, PF and PF/MC, were also investigated. The *in vitro* cytotoxicity of pure PF, PF/MC gels towards MC3T3-E1, a murine osteoblast cell line and also C2C12, a murine myoblast cell line, was investigated. The alkaline phosphatase (ALP) activity, a routine method used to determine cell differentiation, of these gels with and without EDS was evaluated using both cell lines.

## 2. Materials and methods

### 2.1. Materials

Pluronic F127, PBS (phosphated buffer saline pH 7.4), MC (powder form; viscosity 10–25 mPa s for a 2% solution in $H_2O$ at 20 °C; methoxyl content: 27.5–32%), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent, Triton X-100, p-nitrophenylphosphate, zinc acetate and EDS were from Sigma Co., St. Louis, MO, USA. Mouse osteoblastic cells (MC3T3-E1) and C2C12 cells were obtained from the ATCC, Manassas, VA, USA. All other chemicals (magnesium chloride, NaOH, carbonate buffer *etc.*) were all reagent grades obtained from RCI chemicals.

### 2.2. Preparation of samples

The PF solutions were prepared at various concentrations (16, 18 and 20% w/w) using a cold method as previously described (Lin et al., 2004). Briefly, required amount of PF was dissolved in MilliQ water at 4 °C and refrigerated overnight to ensure that the polymer was thoroughly dissolved. For the PF/MC blends, MC was separately dispersed in distilled water with continued stirring at a cold temperature to produce 4% w/w solution. The appropriate amount of PF was then dispersed in the MC solution to obtain 12, 14, 16, 18 and 20% w/w of PF in MC (4% w/w) and these blends were referred to as 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC, respectively. The resulting combination was mixed thoroughly and refrigerated until the blend was completely dissolved.

This sample preparation method was also employed to prepare the samples for the determination of cytotoxicity. However, the PF and MC powder were UV sterilized before use and sterilized water was employed in these sample preparations as previously described (Li et al., 2014). The sample solutions, 0.2 g equivalence of PF or PF/MC, were added to a 24-well plate. After the samples formed a gel, PBS (1 mL) was added to the well, thus the concentration of the PF or PF/MC blend was 0.2 g/mL. The 24-well plate was incubated for 24 h and the PBS extract was filter sterilized. These extract solutions (100 μl) were further used for the cytotoxicity study (Jamuna-Thevi et al., 2011).

EDS has been previously found to be nontoxic at concentrations that ranged from $10^{-4}$ to $10^{-5}$ M to the various cell lines (Itoh et al., 2003; Tanaka et al., 2013). In this study, the pure drug solution at $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$ M of EDS were used to determine the concentration-dependent cytotoxicity of EDS on MC3T3-E1 and C2C12 cell lines. These solutions were prepared by dissolving appropriate amounts of EDS in water. For the gel samples containing EDS, the required amount of the drug to obtain the final concentration of $4 \times 10^{-3}$ M EDS, was dispersed to the 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC solutions, the mixtures were thoroughly mixed until the drug was completely dissolved and the mixtures were referred to as 12PF/MC/EDS, 14PF/MC/EDS, 16PF/MC/EDS, 18PF/MC/EDS and 20PF/MC/EDS, respectively.

### 2.3. ATR-FTIR measurement

ATR-FTIR spectra were recorded using a PerkinElmer Spectrum One spectrometer, equipped with an attenuated total reflectance (ATR) sampling device containing a ZnSe crystal. The spectra were scanned over the range of 4000–650 $cm^{-1}$ using 64 scans at a resolution of 2 $cm^{-1}$. Prior to each scan, a background spectrum of water was scanned using the same parameters. The samples in the sol state were measured immediately after taking the samples from the refrigerator. The samples in the gel state were collected after incubating the sample at 37 °C. All samples remained in the same states before during and after measurements. Deconvolution of the FTIR spectra over the range from 1390 to 1366 $cm^{-1}$ was performed using the GRAMS/AI (7.01) software (Thermo Galactic, Salem, NH, USA) by fitting the spectra with a Gaussian function.

### 2.4. Cell cultures

MC3T3-E1 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum (FBS, Gibco) and antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin, Gibco). To increase the cell numbers, cells were replaced with fresh medium every 2 days. The cultures were kept in an incubator (5% $CO_2$ and 95% air) at 37 °C. After the required confluence was reached, they were cultured for further studies.

### 2.5. Evaluation of cytotoxicity

Cytotoxicity was determined using the MC3T3-E1 and C2C12 cell lines (Ebisawa et al., 1999). The cells were plated at a density of $2 \times 10^4$ cells/well in 96-well plates. The cells were incubated in DMEM at 37 °C, 5% $CO_2$ for 24 h. The media was removed and the cells were washed twice with PBS solution (100 μL). The wells were inoculated with 100 μL of the extract solutions of 20PF and the blends including 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC (see Section 2.2) and incubated in DMEM at 37 °C, 5% $CO_2$ for 24 h. Zinc acetate (100 ppm) and the cell culture medium, were used as a positive and negative control, respectively (Laovitthayanggoon et al., 2009). Media were removed and washed with 100 μL of PBS twice and the PBS was then removed. The MTT reagent 100 μL (0.5 mg/mL) was then added, and the cells were incubated for 4 h at 37 °C (Jamuna-Thevi et al., 2011). The MTT reagent was removed and 100 μL of the solubilizing reagent, dimethyl sulfoxide, was added to dissolve the purple crystals. The quantity of this colored formazan product, was directly proportional to the number of viable cells, and was measured by recording the absorbance at 570 nm using a microplate reader (Beckmans coulter, CA, USA). The cell viability was calculated by comparing the absorbance of the sample with that of the control. This same procedure was also employed for determination of the concentration-dependent cytotoxicity of EDS.

### 2.6. Osteogenic differentiation of the prepared gels

PF and MC powders were UV sterilized and their solutions were prepared using sterile water. The sample solutions (0.5 mL) were added into 24 well plates and allowed to solidify at 37 °C. MC3T3-E1 or C2C12 cells at a density of $2 \times 10^4$ cells/mL were added on to the solidified gels and incubated. The ALP activity was determined using para-nitrophenylphosphate (pNPP) as a chromogenic phosphatase substrate (Wu et al., 2011). Cells in the culture medium were used as a control. The ALP tests were determined after 1, 3, 5 and 7 days of cell culture on the gels. To perform the test, the culture medium was removed and the cells were rinsed twice with PBS. The cells were then lysed by adding 250 μL of Triton X-100 (0.01% in PBS) to each well and incubated for 30 min at 4 °C. The mixture from each well was then transferred to an eppendorf tube and centrifuged at 12000 × g at 4 °C for 15 min. The supernatant (100 μL) was transferred to a new well plate. 500 μL of 20 mM pNPP which had been previously diluted with 250 μL of 0.1 M carbonate buffer (pH 10.2) and 250 μL of 4 mM $MgCl_2$ was then added to each well. The mixture was incubated at 37 °C for 30 min. Subsequently, the reaction was stopped by adding 2 mL of 0.25 N NaOH to the mixture and the absorbance was measured at 410 nm using a microplate reader. The percentage of ALP activity was calculated by comparing the absorbance of the sample with that of the control.

### 2.7. Sol–gel phase transition

The sol–gel transitions of the 20PF and PF/MC blends were observed by the test tube inversion method as previously described (Li et al., 2014; Yu et al., 2008). Briefly, 1 mL of a cold sample was placed in a test tube and placed in a water bath at 20 °C. A sample was heated via stepwise temperature increment, and data were recorded for every 1 °C increase in temperature from 20 to 40 °C. The transition temperature was determined by the absence of a flow when the test tube was inversed for 1 min.

### 2.8. Viscosity of the samples

The rheological measurements were carried out by stress-controlled rheometer (Gemini HR-nano, Malvern Instruments, UK) with a cone-plate geometry (diameter 4 cm, angle 1°). The periphery of the samples was covered by a layer of silicone oil to prevent evaporation. Flow behavior was evaluated to measure the viscosity as a function of the shear rate.

### 2.9. In vitro degradation of polymers

The *in vitro* degradation of the PF and PF/MC blends was carried out by the gravimetric method as previously described (Li et al., 2014). The initial weight of a 2 mL eppendorf tube was weighed ($W_v$). One mL of PF or PF/MC blends were placed in the tube and kept in an incubator for 30 min at 37 °C. Samples were then weighed accurately and the initial weight of the eppendorf tube and gel ($W_i$) were recorded. Subsequently, 2 mL of PBS was added into the eppendorf tube which was incubated at 37 °C .The buffer solutions were removed at regular intervals and hydrogels were weighed ($W_t$). All experiments were performed in triplicate. The percentage weight of the remaining gel was expressed as (Li et al., 2014):

$$\frac{W_t - W_v}{W_i - W_v} \times 100\%$$

### 2.10. Environmental scanning electron microscope.

The PF or PF/MC samples were lyophilized cautiously to preserve their three-dimensional porous structure. Lyophilized samples were immersed in liquid nitrogen, and the vitrified samples were carefully cut with a cold knife. The cut samples were mounted, sputter coated with gold, and their morphology determined by ESEM, Quanta 400 (FEI Czech Republic) using a large field detector in a low-vacuum mode and an accelerating voltage of 15 kV.

### 2.11. In vitro drug release

The release of EDS from the gel of the PF and PF/MC blends was studied using a membrane less diffusion method as reported previously (Yang et al., 2009). The concentration of EDS in all samples was 1 mg/mL ($3 \times 10^{-3}$ M). The solution of 20PF/EDS or PF/MC/EDS blends was placed in a flat-bottomed vial (internal diameter about 18 mm) and the solution was incubated at 37 °C for 5 min for gel formation. Subsequently, 8 mL of physiological saline (0.9% NaCl w/w) was gently placed on the surface of the gels. At predetermined time points, 2 mL of release media was removed and replenished with an equal volume of the fresh medium. The release profiles of EDS were analyzed using a UV spectrophotometer (Cary 60, Agilent Technologies, USA) at a wavelength of 240 nm using a Cu-complexation method by reacting with $CuSO_4$ at an acidic pH (2.8) of $HNO_3$ as previously described (Czuryszkiewicz et al., 2005; Koba et al., 2008; Ostović et al., 1993).

### 2.12. Statistical analysis

Data were reported as the mean ± SD. Statistical analysis was performed using the IBM SPSS Statistics version 22 for Windows.

Data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey *post hoc* test for multiple comparison tests.

## 3. Results and discussion

### 3.1. Sol–gel phase transition

The gel formation temperatures were evaluated by observing the changes in the range of 20–40 °C. The formation of the PF gel with increasing temperature was due to progressive dehydration and the forming of micelles and gel (Li et al., 2008). PF concentrations lower than 16% w/w (12PF and 14PF) did not form a gel and this agreed with previous findings (Sharma and Bhatia, 2004). The 16PF, 18PF and 20PF formed gels at 29, 28 and 25 °C, respectively. This demonstrated that increasing the concentration of PF decreased the sol-to-gel transition temperature. This was consistent with a previous study (Sharma and Bhatia, 2004). The blends of 12PF and 14PF with 4% w/w MC (12PF/MC and 14PF/MC) did form gels. The sol-to-gel transition temperatures for the blends 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC were at 33, 27, 25, 24 and 23 °C, respectively. Increasing the concentration of PF in the PF/MC blends also decreased the sol-to-gel temperatures but they were all slightly lower than for the pure PF. MC is a so-called heat set gel, it becomes a gel at a higher temperature and a sol at a lower temperature. Upon heating, a cool solution of 4% w/w MC demonstrated its sol-to-gel temperature at 52 °C. Based on these evaluations, the blending of MC with PF led to a lowering of the transition temperature of MC from 52 °C. Furthermore the lower gelation temperature of the PF/MC blends compared to the pure PF most likely involved some specific mechanisms. This was probably caused by an increasing interaction between the PF and the MC and a reduction of the intermolecular hydrogen bonding between the PF and water as well as for the MC and the water molecules. A reduction of the water activity of the system may decrease the sol-to-gel transition temperature as previously described when sodium chloride was added to PF (Pandit and Kisaka, 1996). The sol-to-gel transition temperatures of 12PF/MC/EDS, 14PF/MC/EDS, 16PF/MC/EDS, 18PF/MC/EDS, 20PF/MC/EDS were 34, 27, 25, 25 and 25 °C, respectively. There was not much difference in the gelation temperatures for the gels loaded with EDS when compared to the unloaded gels. Thus EDS may not have much interaction with the structures of the PF/MC systems.

### 3.2. ATR-FTIR studies

ATR-FTIR was employed to determine the mechanism of gel formation. As shown in Fig. S1, the whole FTIR spectrum of the aquesous MC showed absorption bands at 3305 $cm^{-1}$ (O—H stretching), 1638 $cm^{-1}$ (C—O) and 1055 $cm^{-1}$ (C—O—C stretching) that were similar to those previously described (Tang et al., 2010a). The characterstic peaks of PF in an aqueous solution at 3400 $cm^{-1}$ (O—H stretching), 1085 (C—O—C stretch) were also observed as previously described (Su et al., 2002). The FTIR spectra of the blend of 20PF/MC and 20PF in a sol state were similar, probably because of the higher amount of PF in the blend.

The spectra of the 18PF/MC and 20PF/MC (representing the PF/MC blends) in the sol and gel states are shown in Fig. 1. Su et al. (2002) described two bands from the $CH_3$ symmetrical deformation mode of PF, one at the higher (1378 $cm^{-1}$) and the other at lower wavenumbers (1373 $cm^{-1}$) that corresponded to the hydrated (sol) and dehydrated (gel) states, respectively. As the temperature approached the critical micellization temperature, the shift towards lower frequencies (1373 $cm^{-1}$) was caused by a reduced interaction between the methyl groups and the water molecules as the methyl groups were experiencing a less polar environment. In this study the PF/MC blends in the sol and gel states retained the characteristics of the pure PF. The $CH_3$ symmetrical deformation peak of the blends in the gel state was observed as a prominent peak but the blends of 20PF in the sol state only displayed one broad peak in the range from 1390 to 1366 $cm^{-1}$ (Figs. 1 and S1). In addition to increasing the temperature, the MC could also induce a less polar environment that reduced the gelation temperature as metioned in Section 3.1. The peak fitting of 20PF/MC blend demonstrated a higher area ratio of the dehydrated band (1373 $cm^{-1}$) to the hydrated band (1378 $cm^{-1}$) when the sample was in the gel state compared to that in the sol state (inset of Fig. 1). This result agreed with the peaks for hydrated and dehydrated $CH_3$ groups in a sol and gel state

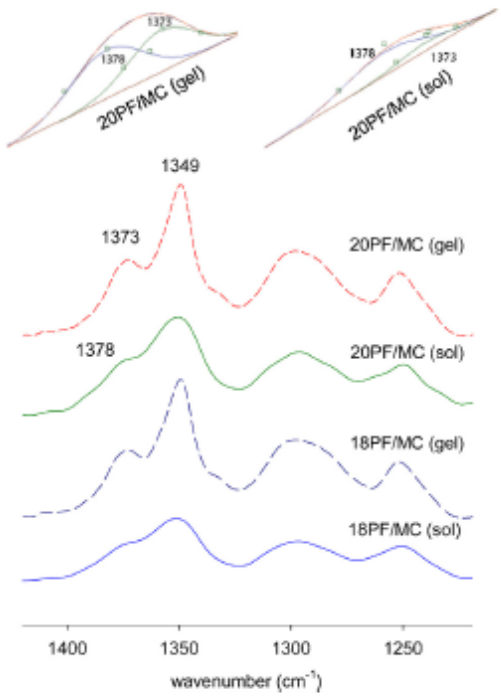


**Fig. 1.** ATR-FTIR spectra of the blends of various concentrations of 18 and 20% (w/w) PF and 4% (w/w) MC in the sol and gel state: in the range from 1420 to 1220 $cm^{-1}$. The fitting peaks of the 20PF/MC gel and sol were performed in the range of 1410–1360 $cm^{-1}$.

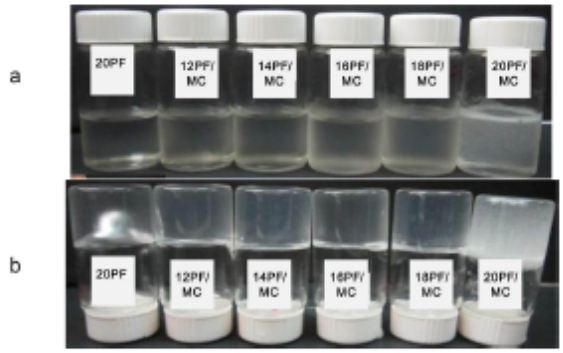


**Fig. 2.** Sol-gel phase transitions of the samples in (a) a sol state at 25 °C and (b) gel state at 37 °C of 20PF, 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC.

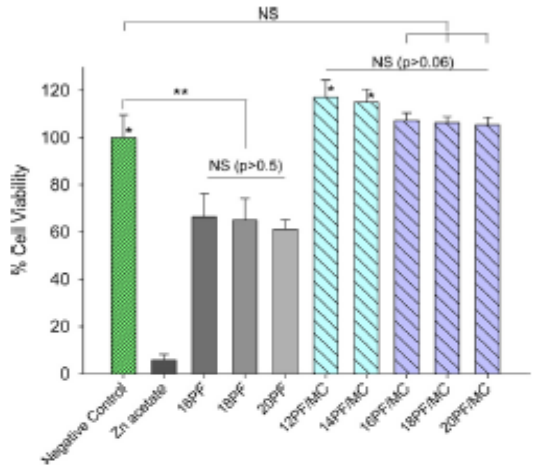


**Fig. 3.** Cytotoxicity of various concentrations of PF and the blends of PF and 4% (w/w) MC on MC3T3-E1 cells. Cytotoxicity of positive (cell culture medium) and negative (100 ppm zinc acetate) controls are also shown. Data are shown as mean ± SD, $n = 8$ per group; $^{*}p < 0.05$, $^{**}p < 0.001$.

as previously described. In addition these FTIR spectra were able to distinguish the sol and gel state of the blends.

### 3.3. Cytotoxicity

Cell viability and cytotoxicity were evaluated using the tetrazolium dye assay on the MC3T3-E1 and C2C12 cell lines. The samples that formed gels at a physiological temperature (37 °C), 16PF, 18PF, 20PF, 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC (Fig. 2), were further tested for their cytotoxicity. Marked differences in the cytocompatibility of the PF and PF/MC on MC3T3-E1 cell lines were observed (Fig. 3). The percentage of cell viability with the 16PF and 18PF was about 65% and were not significantly different from each other but were very much lower than that produced by the negative control ($p < 0.001$). The 20PF gel had the highest toxicity and the cell viability was only 61.1%. In contrast to the pure PF, the PF/MC blends showed no cytotoxicity to the MC3T3-E1 cells. The 12PF/MC and 14PF/MC blends allowed for a higher cell viability than the negative control ($p < 0.5$). The percentage cell viability of the control was not statistically different from those of the 16PF/MC ($p > 0.81$), 18PF/MC ($p > 0.89$) and 20PF/MC ($p > 0.97$). The cytotoxicities or cytocompatibilities of the PF and PF/MC blends on the C2C12 cells (Fig. S2) were similar to those on the MC3T3E1 cells. MC may protect and/or stimulate the cells as shown in previous findings (Kuchler et al., 1960; Mizrahi and Moore, 1970) and demonstrated an improved cell viability and/or cell proliferation compared to the pure PF. The cell viability of the blends increased slightly with lower PF concentrations in the blends but they were not statistically different.

It should be noted that in addition to the samples blended with 4% w/w MC, the PFs were also blended with 3% w/w MC. The cell viabilities of the blends of PF and 3% MC on MC3T3-E1 cell lines were not statistically different from that from the control. However, the blends of PF with 4% w/w MC especially 12PF/MC and 14PF/MC showed a better cell viability than the control (Fig. S3). The blends of PF and 4% w/w MC were therefore chosen for further use in this study.

According to the MTT test, EDS solutions of less than $10^{-3}$ M were non-toxic to both MC3T3-E1 and C2C12 cells as shown in Fig. 4A and S4, respectively. The concentration of 1 mg/mL

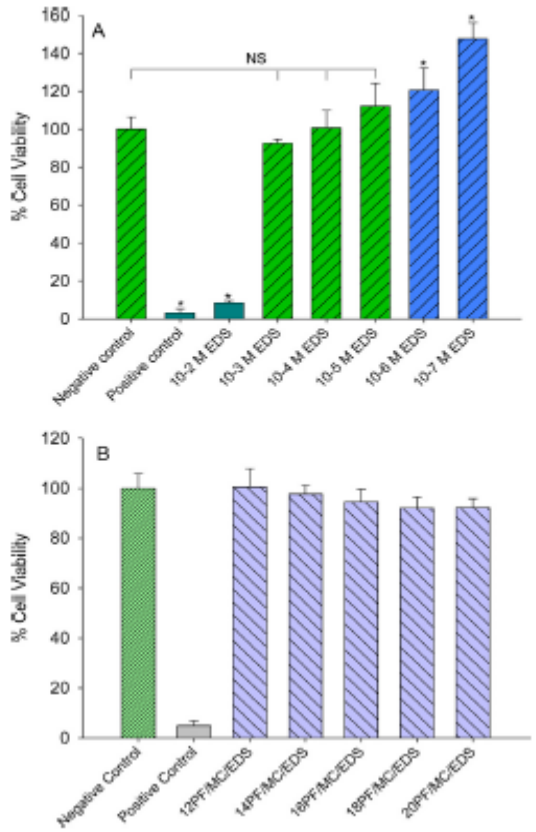


**Fig. 4.** Cytotoxicity of (A) various concentrations of EDS and (B) the blends of $4 \times 10^{-3}$ M EDS, 4% (w/w) MC and various concentrations of PF on MC3T3-E1 cells. Cytotoxicity of positive (cell culture medium) and negative (100 ppm zinc acetate) controls are also shown. Data are shown as mean ± SD, $n = 8$ per group; $^{*}p < 0.001$.

($4 \times 10^{-3}$ M) of EDS was demonstrated to be non-toxic to MC3T3-E1 cells when the drug was incorporated into the PF/MC blends (Fig. 4B). This concentration of EDS was subsequently used for further analyses. When the EDS was loaded at $4 \times 10^{-3}$ M in these blends, the cell viability of the MC3T3-E1 cells of the negative control was not significantly different ($p > 0.6$) from those of the 12PF/MC/EDS, 14PF/MC/EDS, 19PF/MC/EDS, 18PF/MC/EDS and 20PF/MC/EDS (Fig. 4B). Nevertheless, the lower concentrations of PF domonstrated a slightly lower cytotoxicity than the high PF concentrations but were not statistically different.

### 3.4. ALP activity

The ALP activity was determined to obtain an indication of the possibility of stimulation of osteogenic differentiation. The osteogenic effects of all PF/MC and PF/MC/EDS blends that were demonstrated to be nontoxic were determined by the ALP assay over a 7 day period. As shown in Fig. 5A, the ALP activity on MC3T3-E1 cells increased from day 1 to day 7. Higher amounts of PF in the blends resulted in a further increased ALP activity. The 20PF/MC blend produced a significantly higher increase in ALP activity compared to the control ($p < 0.001$). There was no significant difference in the % ALP activity between the 12PF/MC, 14PF/MC and the control after a period of 1 week. However at both day 3 and day 5, an increase in the ALP activity was detected for the 18PF/MC ($p < 0.01$) and 16PF/MC ($p < 0.05$), respectively.

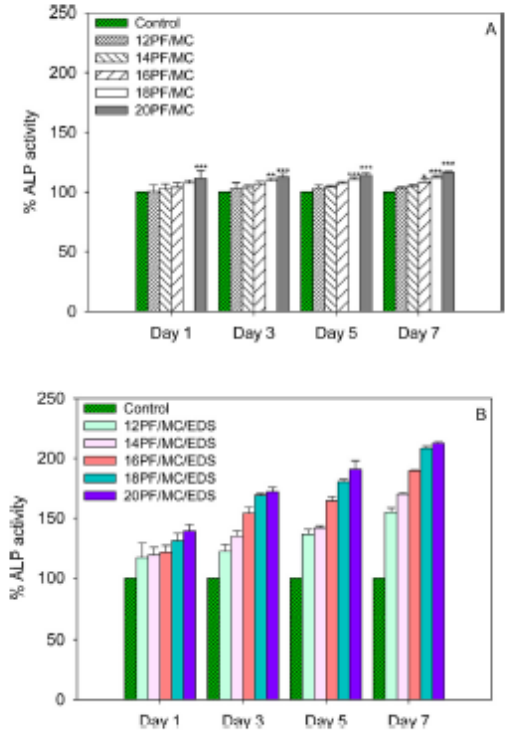


**Fig. 5.** Time course of changes in the ALP activity of various concentrations of PF (A) in 4% (w/w) MC and (B) in 4% (w/w) MC and $4 \times 10^{-3}$ M EDS (EDS) on MC3T3-E1 cell line (mean ± SD, $n = 4$ per group; compare to the control (A) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, (B) $p < 0.001$ for all PF/MC/EDS blends).

As shown in Fig. 5B, the PF/MC/EDS blends produced a highly elevated ALP activity on the MC3T3-E1 cells in a time-dependent manner. For the longer time periods of exposure of the samples to the cells, the ALP activity was significantly enhanced. An extremely high level of ALP activity was detected for the cells grown with the drug loaded gels (PF/MC/EDS) compared to the pure gels (PF/MC) at all times of the study. For example, the increase in ALP activities for the MC3T3-E1 cells cultured for 7 days with 20PF/MC/EDS and 20PF/MC were 53 (212%) and 14 fold (116%), respectively, compared to the control (100%). The ALP activity in the osteoblast precursor cell line (C2C12) was also determined. As shown in Fig. S5, the higher ALP activities were caused by drug loaded gels compared to the pure gels when the C2C12 cells were exposed to the samples. The ALP activity was also enhanced in a time-dependent manner. EDS has been previously reported to increase the activity of the MC3T3-E1 cells (Itoh et al., 2003). These results demonstrated that EDS loaded gels significantly increased proliferation of both osteoblasts (MC3T3-E1) and myoblasts (C2C12). This provided a strong indication for the bone regeneration effect of the PF/MC/EDS blends since the blends also enhanced the murine osteoblast precursor cell lines (C2C12) that do not normally express ALP activity. The expression of the osteogenic markers including a strong induction of ALP activity but an inhibition of myogenic differentiation has been reported when the C2C12 cells were treated with various substances such as bone morphogenetic proteins and inorganic phosphates (Ebisawa et al., 1999; Katagiri et al., 1994; Kikkawa et al., 2009). The mechanisms for these effects are not truly understood and might involve a different pathway (Chen et al., 2004; Nakashima et al., 2005). Since the structure of the EDS is analogous to that for inorganic

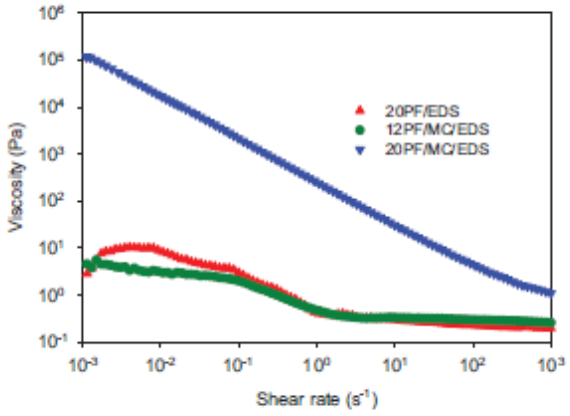


**Fig. 6.** Viscosity–shear rate flow curves for samples containing various concentrations of PF, 4% w/w MC (MC) and $4 \times 10^{-3}$ M EDS (EDS) at 25 °C.

phosphates, a similar mechanism for the osteogenic effect may have occurred. Nevertheless, determination of the exact mechanism will require further studies.

### 3.5. Viscosity of the samples

The flow curves of 20PF/EDS, 12PF/MC/EDS and 20PF/MC/EDS at room temperature are shown in Fig. 6. Increasing concentrations of PF in the blends resulted in an increase of the viscosity when compared 12PF/MC/EDS to 20PF/MC/EDS samples. The viscosity of the 20PF/MC/EDS was significantly higher than the 20PF/EDS thus clearly reflecting the enhancement of viscosity in the presence of MC. Thus both the MC and PF may influence the viscosity of the systems. These samples displayed a pseudoplastic behavior (shear thinning) which viscosity decreased with an increase of the shear rate. This behavior could therefore be employed to advantage in facilitating the flow of the PF/MC/EDS during injection through a syringe for parenteral use.

### 3.6. In vitro degradation

Degradation of the 20PF and PF/MC gels was measured as a function of the incubation time in PBS at 37 °C as shown in Fig. 7. The weight loss gradually decreased with increasing

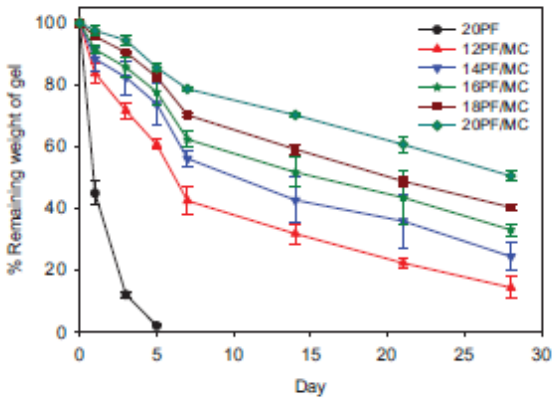


**Fig. 7.** *In vitro* degradation of 20PF and the blends of various concentrations of PF and 4% (w/w) MC in the gel state at 37 °C (mean ± SD, $n = 3$).

concentrations of the PF in the blends. Samples with high concentrations of PF (20PF) were completely degraded within 5 days. This may be due to the gel structure of PF that undergoes a rapid dissolution and disintegration upon incubation in a PBS buffer solution. However the PF/MC gels were not completely degraded after 28 days. The percentage of gels that remained after 28 days was 14, 24, 33, 40 and 50% for the 12PF/MC, 14PF/MC, 16PF/MC, 18PF/MC and 20PF/MC, respectively. As found in a previous investigation, pluronic gels were stabilized in the presence of various polysaccharides (Kjoniksen et al., 2014). This might be due to the interactions between the hydrophobic parts of the polysaccharides and the pluronic gels. Based on the ATR-FTIR studies, PF interacted with MC and increased the hydrophobic environment of the system. This could increase the stability of the PF gels. In addition, the increase of viscosity in the system may also play a role by decreasing the degradation as previously described (Bhardwaj and Blanchard, 1996). As shown in Fig. S6, the viscosity at 37 °C of 20PF increased by about 10–20 fold at different shear rates in the presence of MC (20PF/MC). Therefore, the enhancement of viscosity might also slow the degradation of PF/MC. Consequently, this stabilization made the blends more functional for a sustained delivery of a drug.

### 3.7. Gel morphology

The formation of characteristic structures may result from the formation of the gels. Thus, the morphology of PF/MC blends was examined using ESEM. Fig. 8 shows the SEM micrographs of 20PF and PF/MC xerogels after removal of water by lyophilization. A network with an interconnected porous structure was observed.

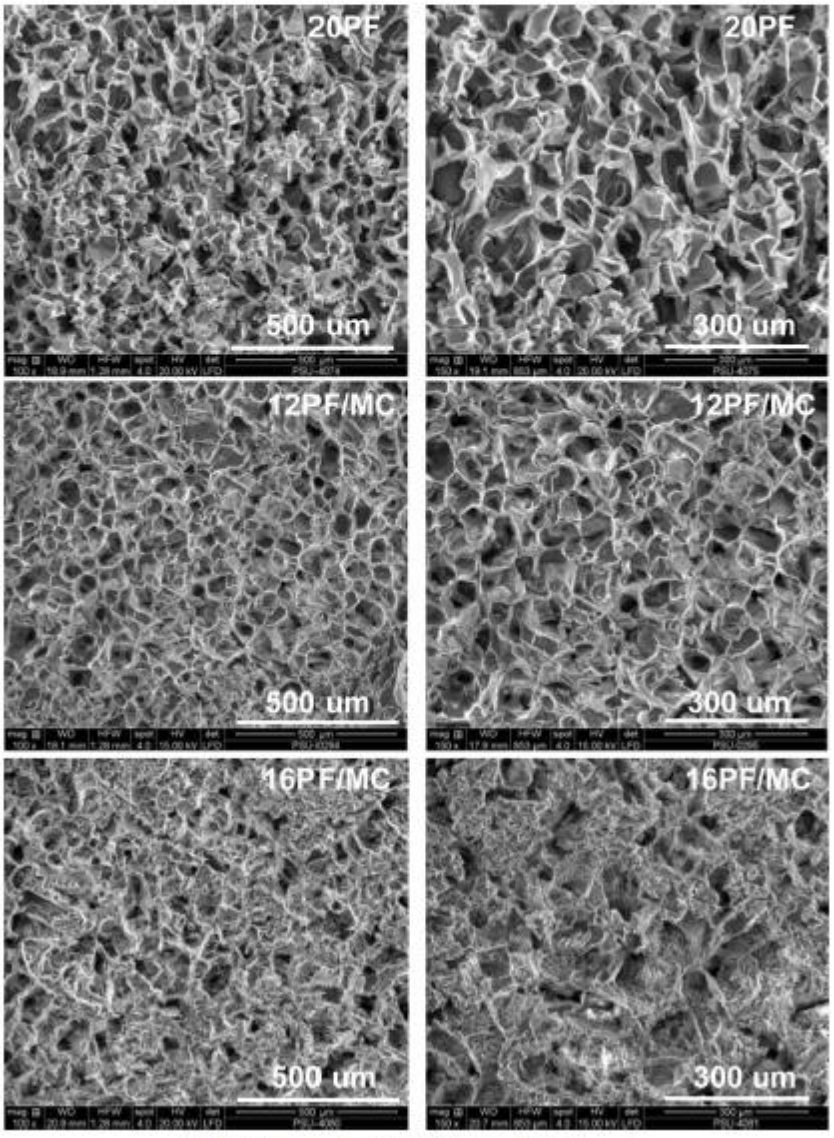


**Fig. 8.** SEM micrographs of 20PF, 12PF/MC and 16PF/MC xerogels.

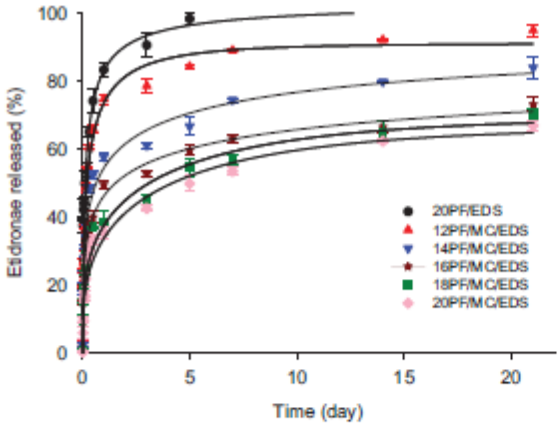


**Fig. 9.** *In vitro* release of EDS from the gels of 20PF and the blends of various concentrations of PF and 4% (w/w) MC and $4 \times 10^{-3}$ M EDS at 37 °C (mean ± SD, $n = 3$).

The surface of 16PF/MC was more dense when compared to that of the 12PF/MC and indicated that a high amount of PF increased the compactness of the blend. The pore sizes of the pure PF (20PF) were bigger than that of PF/MC blends so the presence of MC enhanced the compactness of the blends.

### 3.8. In vitro *release of etidronate disodium*

Previous studies have demonstrated the slow release of hydrophilic drugs such as vancomycin and mitomycin C from a PF gel alone (Miyazaki et al., 1992; Veyries et al., 1999). In this study the release of EDS which is a hydrophilic molecule from PF alone and the PF/MC blends was investigated. An *in vitro* release profile of EDS in the 20PF and all the PF/MC blends was examined at 37 °C. As shown in Fig. 9, there was an initial burst of release from the 20PF and PF/MC gels followed by a sustained release during later days. The 20PF sustained the release of the drug for only 5 days whereas the PF/MC gels prolonged the release for more than 28 days. EDS may be located primarily outside and to a lesser extent inside the micelles as found in a previous study for a hydrophilic molecule such as cephalexin with a log *P* of −1.74 (Moore et al., 2000). The relevant release mechanism for EDS could be due to the entrapment of the EDS on the outer micelles that led to the initial burst release. As shown in Fig. 10, the higher concentration of the PF in the blends produced a more dense internal stucture. These dense structures together with the increase of the viscosity and the slow disintegration (Fig. 7) due to the addition of MC may result in the retarded release of EDS that remained at the outside of the micelles and/or the EDS that had been partially entrapped in the inner core of the gels.

## 4. Conclusion

In this work, PF/MC gels with a better cytocompatibility and improved stability were successfully developed without the use of any objectional chemicals, pH or organic solvents thus enabling them to be readily used for tissue engineering and drug delivery systems. It was interesting that a concentration of PF as low as 12% could gel with the addition of MC. In addition, the viability of MC3T3-E1 and C2C12 cells in the presence PF was significantly increased in the presence of MC. The blends of 12PF/MC, 14PF/MC existed as sols at room temperature and as a gel at 37 °C in addition their viscosities were relatively low at room temperature and thus could ease the flow during injection. These blends may be useful for delivery of an injectable implant drug. Moreover EDS loaded gels produced osteogenic differentiation by MC3T3-E1 cell as well as C2C12 (myoblasts) cells that usually would not express ALP so this demonstrated their significant osteogenic effects. The gels containing MC had a sustained release of EDS for 28 days. Therefore, these gels may be able to sustain the release of EDS when injected at the site of application that would enhance its application in bone remodeling.

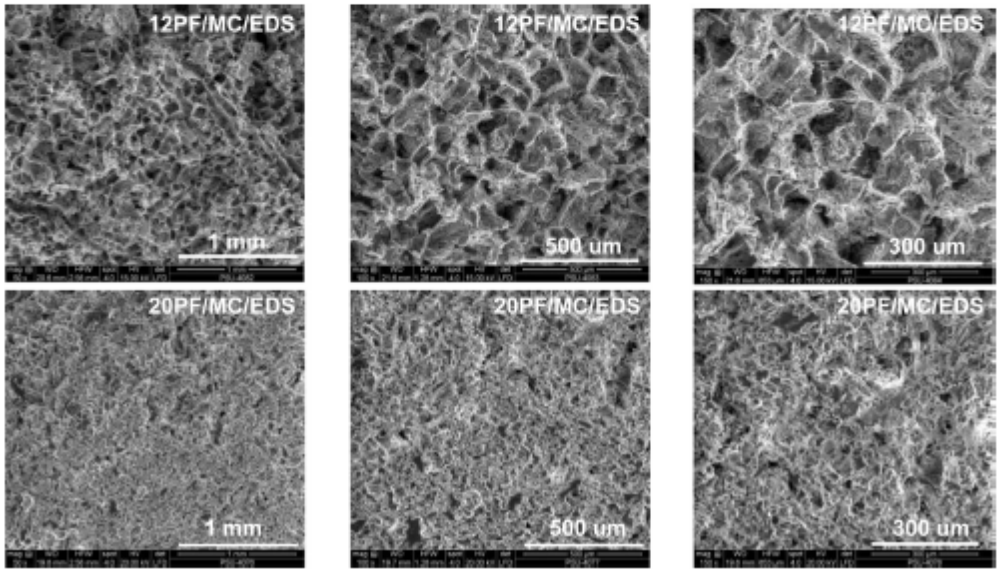


**Fig. 10.** The SEM micrographs of 12PF/MC/EDS and 20PF/MC/EDS exrogels.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2015.12.070.

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# Etidronate Loaded Gelatin Nanoparticles by Nanoprecipitation

AparnaSaiLaxmi RANGABHATLA [1], Vimon TANTISHAIYAKUL [1,2] * & Kwunchit OUNGBHO [3]

[1] *Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90112 Thailand*

[2] *Nanotec-PSU Center of Excellence for Drug Delivery System, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90112 Thailand*

[3] *Center of Excellence for Drug Delivery System, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, 90112 Thailand*

***SUMMARY.*** **A simple and inexpensive approach for preparation of drug loaded nanoparticles by nanoprecipitation has been utilized in the present work. Bisphosphonates are drugs of choice for bone disorders like osteoporosis and Paget's disease. Due to short half life in circulation (0.5-2 h), controlled release of bisphosphonates is essential for long term therapeutic efficacy. Etidronate, a bisphosphonate, was selected as a model drug in this study. Gelatin-etidronate nanoparticles were successfully prepared by nanoprecipitation method. Polymeric nanoparticles play a vital role in entrapping drug molecules and slow the release of the drug. The nanoparticles were characterized by Zetasizer and TEM for their size and morphology, respectively. The prepared nanoparticles were in the size range of 100-200 nm. The zeta potential value was found to be +5.5 mV. The drug loading efficiency was found to be 40%. *In vitro* drug release studies revealed control release of the drug from the polymer matrix. These nanoparticles exhibited dose dependent cytotoxicity on mouse C2C12 cells.**

*RESUMEN*. En el presente trabajo se ha desarrollado un enfoque simple y de bajo costo para la preparación de nanopartículas cargadas con fármaco por nanoprecipitación. Los bisfosfonatos son fármacos de elección para los trastornos óseos como la osteoporosis y la enfermedad de Paget. Debido a la corta vida media en circulación (0,5 a 2 h), la liberación controlada de los bifosfonatos es esencial para la eficacia terapéutica a largo plazo. El etidronato, un bisfosfonato, fue seleccionado como medicamento modelo en este estudio. Nanopartículas de gelatina etidronato se prepararon con éxito por el método de nanoprecipitación. Las nanopartículas poliméricas desempeñan un papel vital en atrapar moléculas de fármacos y ralentizan la liberación del fármaco. Las nanopartículas se caracterizaron mediante Zetasizer y TEM para su tamaño y morfología, respectivamente. Las nanopartículas preparadas estaban en el rango de tamaño de 100-200 nm. El valor del potencial zeta se encontró que era 5,5 mV. La eficiencia de carga de fármaco se encontró que era 40%. Los estudios de liberación de fármaco *in vitro* revelaron la liberación controlada del fármaco desde la matriz polimérica. Estas nanopartículas exhiben citotoxicidad dosis-dependiente en células C2C12 de ratón.

## INTRODUCTION

Etidronate, a model drug in this study, comes under the class of bisphosphonates that are stable analogs of pyrophosphate that regulates calcification and bone resorption. Basically the affinity of the bisphosphonates to hydroxyapatite is primarily responsible for inhibition of ectopic calcification and thus bone resorption. Therefore, bisphosphonates are drugs of choice for many bone related diseases such as osteoporosis and Paget's disease. Etidronate is also highly water soluble. The major problem of etidronate is its relatively short half life in circulation (0.5-2 h) so gelatin based biocompatible nanoparticles will provide an alternative for conventional treatment making a sustained release of the drug. However, the very low oral absorption (less than 1%) and relatively short half life in circulation (0.5-2 h) are the major issues pertaining to long term and sustained delivery to bone related disorders [1].

*KEY WORDS:* drug delivery, etidronate, gelatin, nanoparticles, nanoprecipitation.

* Author to whom correspondence should be addressed. *E-mail:* vimon.t@psu.ac.th



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Polymeric nanoparticles (NPs) have numerous advantages which include relatively high intestinal uptake probably due to charge properties and particle size. Extensive advantages of gelatin such as low antigenicity, availability of functional groups that are easily accessible and provide many opportunities for pairing with crosslinkers and targeted ligands, make it a desirable biomaterial. Moreover gelatin is widely used as a stabilizer in vaccines and also in parenteral formulations. In this study, the NPs were prepared by a simple, rapid instantaneous method that used for preparation of NPs from different polymers as it does not require any kind of comprehensive treatment such as high temperature, usage of harsh chemicals/solvents and sonication [2]. Gelatin type A is basic in nature with many amino groups which will entrap etidronate that is negatively charged to give a condensation product. Type of gelatin is chosen based on the capability to entrap the drug molecule. The aim of the present work is to perform preliminary studies on cytotoxicity and release properties of prepared NPs.

## MATERIALS AND METHODS

Etidronate disodium is purchased from TCI Chemicals and gelatin type A from Sigma Aldrich. The NPs were prepared by nanoprecipitation as previously described 2. Briefly, 5 mg of etidronate was added to 2 mL of distilled water and to this 40 mg of gelatin (2%) was added and stirred for 30 min at 50 °C temperature (solution A). Solution A is then carefully added to 30 mL of ethanol containing 1.28 g of pluronic F127, an emulsifier. The pluronic F127 to gelatin mass ratio was 32:1. The colloidal solution was then centrifuged and freeze dried. Blank gelatin NPs were also prepared by same procedure without the drug. The freeze dried samples were weighed and the yield of NPs obtained was calculated [2]. Blank particles are calculated similiarly. The drug loading efficiency was determined using Eq. [**1**] [3].

$$Drug\ loading\ efficiency = \frac{Total\ amount\ of\ drug\ -\ Free\ drug}{Total\ amount\ of\ drug} \times 100 \quad [1]$$

The zeta potential, size distribution and polydispersity index (PDI) was measured using zetasizer (Zetasizer Nano ZS90, Malvern, UK). Furthermore the morphology of particles was analyzed by TEM (TEM JEOL-2010). *In vitro* drug release was performed as described previously: briefly, 5 mg of NPs were placed in 5 mL of PBS 7.4 in dialysis membrane (14,000 to 20,000 KDa) and this membrane was immersed in 50 mL of the release medium. The experiment was carried out at 37 °C at 40 rpm in a shaking incubator (Diahan Labtech Co. Ltd). Sample (2 mL) was withdrawn at regular intervals and replaced with fresh PBS (pH 7.4).The samples were analyzed by copper complexation method at 240 nm as previously described [4].

Preliminary biocompatibility of the prepared NPs was performed on C2C12 cell lines by MTT assay. Cells were seeded at a density of $2 \times 10^4$ cells/well in 96-well plates and incubated at 37 °C for 24 h. Media was removed and plates were washed thoroughly with PBS and wells were inoculated with different concentrations of etidronate in the gelatin-etidronate NPs ranging from 50 to 250 µg/mL. Subsequently, the cell viability was determined by measuring the absorbance at 570 nm using microplate reader [5].

## RESULTS AND DISCUSSION

Gelatin has been chosen for the preparation of NPs based on its ability to form colloidal nanoparticle suspensions with simple technique in addition to its biocompatibility, biodegradability and relatively low antigenicity [2]. Specifically the conjugation of water soluble polymers to bisphosphonates, is a promising approach for biomaterials to approach bone tissues [6]. The present work aimed at sustained release of etidronate from gelatin-etidronate NPs. The prepared NPs were characterized by zetasizer for particle size, homegeneity (PDI) and surface charge (zeta potential). The mean size of unloaded NPs was 107.2 nm and that of etidronate loaded gelatin NPs was 262 nm. The mean size of etidronate loaded gelatin NPs was slightly higher than that of gelatin NPs [2]. Particle size of the NPs is dependent on the concentration of ethanol; the higher the concentration of ethanol, the lower the particle size. In our experiments we have used 90% ethanol in order to achieve minimum solubility of gelatin and etidronate [7]. The zeta potential values and PDI values were given in Table 1.

Generally, the lower PDI values indicated the homogeneity of the dispersion. Due to the

| Parameter | Gelatin nanoparticles | Etidronate loaded gelatin nanoparticles |
|---|---|---|
| Mean particle size | 107.2 (nm) ± 3.27 (nm) | 262(nm) ± 5.64 (nm) |
| Zeta potential | +5.86 mV | +1.98mV |
| Polydispersity index | 0.231 ± 0.005 | 0.509 ± 0.005 |

**Table 1**. Mean size, zeta potential and polydispersity index of gelatin and gelatin-etidronate nanoparticles.

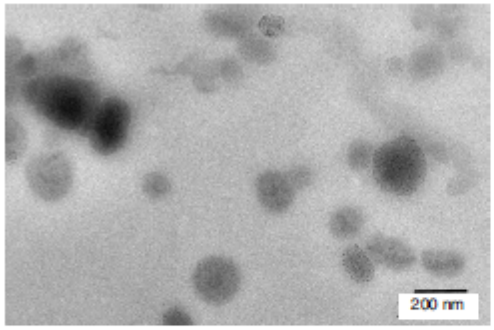


**Figure 1**. TEM image of gelatin-etidronate nanoparticles.

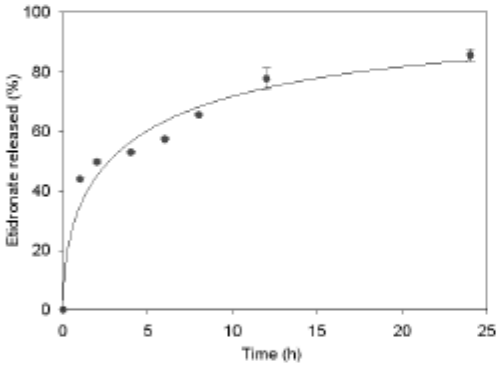


**Figure 2**. *In vitro* release of etidronate from gelatin-etidronate nanoparticles.

high ethanol concentration used in this study, the PDI is slightly high [7]. In addition, Because of interaction between positively charged gelatin and negatively charged etidronate there is difference in zetapotential values between the NPs and etidronate loaded NPs. Pure gelatin nanoparticles with unoccupied aminogroups showed higher positive charge than the drug loaded nanoparticles. The TEM micrograph (Fig. 1) showed that the NPs are spherical with a relatively smooth surface [3].

The *in vitro* release profile of etidronate in

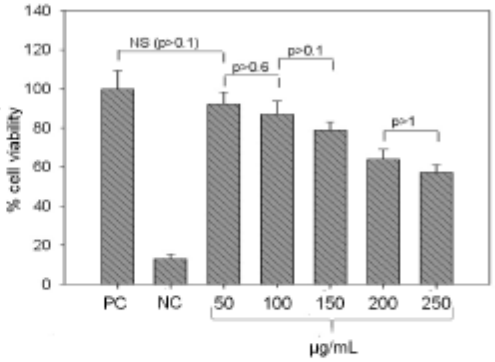


**Figure 3**. Cytotoxicity of gelatin-etidronate nanoparticles at various etidronate concentrations.

PBS 7.4 is shown in Fig. 2. The release of etidronate from gelatin NPs was found to be in a sustained manner. The initial burst release may be attributed to the etidronate that was accumulated at the surface of NPs that could easily diffuse to the external medium [3]. The percentage release from the NPs increased consistently and reached a plateau at about 12 h. The modified release of etidronate may be attributed to the polyelectrolyte complexation between the acid groups of etidronate and the amine groups of gelatin protein matrix [2].

The cytotoxicty of gelatin-etidronate NPs significantly increased at a concentration of 200 µg/mL (Fig. 3). However the NPs increased the biocompatibility of etidronate to a greater extent since etidronate is cytotoxic at a concentration less than 2.5 µg/mL (100 µM) to many cell lines [5].

In conclusion, gelatin-etidronate NPs were successfully prepared by nanoprecipitation method with satisfactory drug loading efficiency. The low PDI values (< 1) indicated homogeneity of the NPs. The NPs exhibited slow release without much burst release. The cytotoxicity of etidronate was markedly reduced in the gelatin NPs, thus enhancing the biocompatibility of etidronate. Hence gelatin-etidronate NPs are promising means for bone related disorders.

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# VITAE

**Name** Mrs. Rangabhatla S.L. Aparna

**Student ID** 5510730009

**Educational Attainment**

| **Degree** | **Name of Institution** | **Year of Graduation** |
|---|---|---|
| Bachelor of Pharmacy | Andhra University | 2003 |
| Master of Pharmacy | Dr M.G.R.Medical University, India | 2006 |

**Scholarship Awards during Enrollment**

**1.**Thesis Grant, Education fee and salary .

The Thailand Education, Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

2.Conference Scholarship

Faculty of Pharmaceutical Sciences, Prince of Songkla University.

**Work-Position and Address**

Dy. Manager,

INM technologies Pvt Ltd (shilpa medicare)

Department of Biomedical & Pharmaceutical Technology

#4,12KM Industrial estate, Kenchanhalli, R.R.Nagar

Bangalore 560098

List of Publications and Proceedings

Paper 1 Rangabhatla, Aparna Sai Laxmi, Vimon Tantishaiyakul, Kwunchit Oungbho, and Onpreeya Boonrat. "Fabrication of pluronic and methylcellulose for etidronate delivery and their application for osteogenesis." *International Journal of Pharmaceutics* 499, no. 1-2 (2016): 110-118.

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