Pharm Res (2015) 32:3149–3158
DOI 10.1007/s11095-015-1692-4

RESEARCH PAPER

Design and Development of Bioceramic Based Functionalized PLGA Nanoparticles of Risedronate for Bone Targeting: *In-vitro* Characterization and Pharmacodynamic Evaluation

Purnima Rawat[1] • Kapil Manglani[2] • Sarika Gupta[2] • Abul kalam[3] • Divya Vohora[3] • Farhan Jalees Ahmad[1] • Sushama Talegaonkar[1]

Received: 9 February 2015 / Accepted: 30 March 2015 / Published online: 4 April 2015

ABSTRACT

Purpose Bioceramic(Hydroxyapatite) based Poly(D,L-lactide-co-glycolide) (PLGA) and polyethylene glycol (PEG) nanoparticles of Risedronate was prepared by dialysis method for bone-targeting.

Methods Risedronate, a targeting moiety that has a strong affinity for bone, was conjugated to PLGA *via* carbodiimide chemistry. Mono-methoxy PEG(mPEG)-PLGA block polymers were synthesized and used to impart surface hydrophilicity to nanoparticles to avoid its uptake by reticuloendothelial system (RES). The structure of prepared di block copolymers were characterized by FT-IR and NMR spectrometry. Risedronate was adsorbed on the surface of hydroxyapatite (RIS-HA) and it was conjugated with different ratios of mPEG-PLGA. The formation of surface-modified PLGA nanoparticle prepared with various ratios of risedronate as well as hydroxyapatite and mPEG was confirmed by ^{1}H NMR and FT-IR spectrometry.

Results Size and % entrapment of the prepared nanoparticle was found to be 79.3 ± 2.3 nm and 93 ± 3.1%. Transmission electron microscopy (TEM) revealed that mPEG-PLGA-RIS-HA nanoparticles possess smooth and uniform surface. Pharmacodynamic study was performed on Dexamethasone (DEX) induced osteoporotic model. The effect of various formulations (mPEG-PLGA-RIS, mPEG-PLGA-RIS-HA and RISOFOS tablet) on bone was studied by Volume bone density (VBD) and by histopathological evaluation. Interestingly mPEG-PLGA-RIS-HA, showed a significant enhancement in bone micro-architecture when compared with other formulations.

Conclusions The results strongly implicated that mPEG-PLGA-RIS-HA has a therapeutic benefits over risedronate sodium monotherapy for the treatment of osteoporosis in a rat model.

KEY WORDS bioceramic · bone density · nanoparticle · osteoporosis · risedronate

ABBREVIATIONS

CaP	Calcium phosphate
DCC	Dicyclohexyl carbodiimide
DEX	Dexamethasone
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
HA	Hydroxyapatite
mPEG	Methoxy polyethylene glycol
NHS	N-hydroxyl succinimide
PLGA	Poly(D,L-lactide-co-glycolide)
RIS	Risedronate
VBD	Volumetric bone density analysis

INTRODUCTION

Osteoporosis or porous bones is a disease characterised by low bone mass and structural deterioration of tissue leading to bone fragility and an increased susceptibility to fractures of the hip, spine, wrist and other bones. Such fractures are associated with mortality and with significant short- and long-term morbidity (1). According to the International Osteoporosis Foundation (IOF), osteoporosis affects 75 million people in Europe and Japan and over 200 million people worldwide, 80% of which are women (2). The studies investigating the prevalence of fractures in this population are scarce, an prevalence of vertebral fractures in young (<35 years) of 3 per 100,

✉ Sushama Talegaonkar
stalegaonkar@gmail.com

[1] Nanomedicine Research Lab, Department of Pharmaceutics Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India

[2] Molecular Sciences Lab, National Institute of Immunology, New Delhi, New Delhi 110067, India

[3] Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India

000 persons per year, amounting to 21 per 100,000 person per year in the population aged 35–44 years is approximated (3). Although there is an increased global awareness of osteoporosis, the disease is still under-investigated and under-treated. The treatment of osteoporosis must focus on all the aspects of the condition that are – bone mass should be maximised; fractures should be prevented people who have already sustained a fracture should be rehabilitated to minimise associated pain, limitation of physical activities (2).

Bone is such a unique composite, containing a collagenous hydrogel matrix and on a volumetric basis it consists of about 33–43% apatite minerals (in-organics), 32–44% organics, and 15–25% water (4). Hydroxyapatite (HA) is naturally occurring inorganic component found in the bone matrix and teeth and due to this structural and functional similarity, extensive research efforts have been reported on synthetic HA as a bone substitute for replacement in several biomedical applications. HA does not have negative osteoclast activity but it has a positive bone remodeling or osteogenic activity (5). These properties are very important because bone tissue constantly undergoes remodelling, a process whereby bone tissue is simultaneously replaced and removed by the bone cells, (osteoblasts and osteoclasts, respectively) (6). In recent studies a significant improvements in the action of HA was observed when it was converted to the nano-scale range particles, study conducted by Sun *et al.*, reported the influence of particle size of HA on *in-vivo* osteoblast cells and discovered that the therapeutic response was improved when smaller particle sizes (0.5–3.0 μm) were used.

Among various classes of drugs bisphosphonates offers the most potent types of molecules to treat osteoporosis. Alendronate and risedronate are considered as a first-line therapy for the prevention and management of osteoporosis in postmenopausal women, as well as the treatment of osteoporosis in men (7). Like all bisphosphonates, Risedronate works by targeting farnyl diphosphate synthase (FPPS) and inhibiting protein prenylation (8).

The major obstacle that limits the successful use of orally administered risedronate includes low permeability and more importantly, insufficient and fluctuating bioavailability. This poor bioavailability(<1%) of Risedronate results in the supplementation of high doses that may perhaps lead to severe side effects like Osteonecrosis of the jaws, fever, vein irritation, general aches and pains and kidney dysfunction (9).

Various attempts were made to improve the oral bioavailability of Risedronate based on improving permeability and enhancing dissolution rate. Among these, the use of microparticulate drug delivery system of Risedronate is proved to have significantly enhanced the permeability through the mucous membrane (10). Apart from this, some authors demonstrated that physical modification such as particle size reduction and conversion to amorphous state are found to boost the solubility and bioavailability of bisphosphonate (11, 12). Besides this, the use of polymer–drug conjugates in drug delivery system has started attracting extensive attention due to their particular therapeutic properties, such as extended half-life, improved bioavailability and targeting to specific cells, tissues or organs by attaching a tracking device (13–17). Recent work also reveals that bisphosphonate conjugated with PLGA and modified with both Alendronate and polyethylene glycol (PEG) for parental drug delivery not only helps in strengthening the bone-targeting potential along with prolonged retention of the conjugate in the body but also reduces adverse side effect, all of which led to noticeably improved bioavailability and anti-resorptive efficacy *in vivo* (18). The another study was also revealed that risedronate-hydroxyapatite (RIS-HA) nanoparticle can be for effective management of osteoporosis (19). Nanoparticles used to target bone tissue were developed by PLGA–PEG diblock copolymer as a bone-targeting moieties based on aspartic acid (20) (Fu *et al.*, 2014). The loading of the bisphosphonate zoledronate into a CaP cement has been reported by Kyllonen L *et al.* (21, 22).

Thus, a nano-sized drug delivery system can be a very promising approach of improving drug bioavailability at target site. Therefore, it can be proposed that PLGA modified–drug conjugate along with HA could be used as a technological platform capable of improving absorption of bisphosphonates . In the proposed work, an attempt has been made to design and develop a hydroxyapatite(HA) based nano-conjugates of Risedronate (RIS) with mPEG-PLGA moiety prepared for targeted delivery to the bone and for effective management of osteoporosis. RIS has strong affinity for bone along with HA to create a dual targeting configuration and PEG component was selected to provide hydrophilic surface; both were incorporated into the design of the functional nanoparticles.

MATERIALS AND METHODS

Materials

Risedronate sodium (RIS) was obtained as a gift sample from Jubilent life science (Noida, India). PLGA 50:50 (Resomer® RG 503 H) was obtained from Evonik Research (Banglore, India). mPEG 2000-DSPE (PE 18:0/18:0-PEG 2000) were obtained from Lipoid (Ludwigshafen, Germany). Dicyclohexyl carbodiimide (DCC), N-hydroxyl succinimide (NHS) and stannous octoate, hydroxyapatite (HA) were purchased from Sigma chemicals (India). Dimethylformamide (DMF, purity N99.0%), dimethyl sulfoxide (DMSO, purity N99.0%) and diethyl ether (purity N99.0%) were purchased from SD fine chemicals (India). Dialysis sacs (Mol. wt. cut-off: 12 000 Da, flat with 25 mm, a diameter of 16 mm, a capacity

of 60 mlft) was purchased from Sigma Aldrich Chemicals, St. Louis, MO. All other chemicals used were of analytical grade.

Synthesis of mPEG–PLGA Block Copolymer

The method reported by choi *et al.* was used with slight modification for the synthesis of mPEG–PLGA block copolymer (18). Dehydrated mPEG was used without further purification. Dehyradation of mPEG was done under vacuum at 70°C for 12 h and were used without further purification. Different amount of PLGA (molar ratio 50:50, Resomer® RG 503 H) and mPEG were put into glass ampoules. Stannous octoate (dissolved in toluene) was added at a concentration of 0.03% (w/w) to this mixture. The air was expelled out from the ampoule by vacuum pump at 25°C for 4 h and then hermetically sealed . The polymerization was initated by heating an ampoule in an oil bath at 130°C for 24 h. The obtained polymer was then purified by dissolution in acetone followed by the precipitation in water. Finally, the purified polymers was dried in a vacuum oven at 25°C for 12 h (17). The prepared Block copolymer was further characterized by ^{1}H NMR and FT-IR spectrometry.

Fabrication of HA-RIS Particles

Risedronate-Hydroxyapatite(RIS-HA) particles were prepared by adding plain hydroxyapatite (HA) particles into 100 ml RIS solution in distilled water (0.5 mg/ml). The solution was kept at 37°C under stirring for 24 h (Table I). The deposited phase was isolated and washed three times with distilled water before drying. The amount of drug adsorbed on HA particles was calculated and these particles were further used for conjugation with PLGA. The process yield and % entrapment of Risedronate (RIS) with different ratios of RIS and HA is shown in (Table I).

Conjugation of RIS-HA to PLGA

S.W. Choi *et al.* method with slight modification was used. Briefly, one gm of PLGA was dissolved in 20 mL acetone and activated by 100 mg Dicyclohexylcarbodiimide DCC and 60 mg N-hydroxysuccinimide NHS overnight at room temperature. The by-product of the activation, dicyclohexylurea, was removed using a syringe filter with 0.45 µm pore size. The NHS-activated PLGA was then precipitated in cold diethyl ether and dried at 25°C for 4 h in a vacuum oven. 100 mg of activated PLGA and 100 mg RIS–HA were dissolved in 20 mL of a mixture (19 mL DMSO and 1 mL distilled water) and then stirred for 24 h at room temperature. The conjugated polymer was precipitated in cold diethyl ether and water sequentially, and then dried at 25°C for 4 h in a vacuum oven (18). The conjugate was characterized by ^{1}NMR (400 MHz) and FT-IR spectrometry (Tensor 27, Bruker Biospin, Germany) to confirm the hydroxyl-carboxyl conjugation between risedronate and hydroxyapatite.

Table I Process Yield and Percentage Drug Loading of Risedronate on Hydroxyapatite Nanoparticles

S.No.	Percent drug loading		% RIS Entrapment
1.	RIS-HA Ratio	Process yield	
2.	1:1	83.20 ± 1.3	75 ± 1.02%
3.	2:1	75.6 ± 0.16	98 ± 0.59%
4.	2:3	71.28 ± 0.9	88 ± 0.0%
5.	3:2	88.50 ± 2.1	70 ± 1.0%
6.	3:1	78.0 ± 5.4	74 ± 1.09%
7.	1:2	90.55 ± 2.7	77 ± 0.6%
8.	5:4	88.28 ± 0.6	85 ± 0.44%
9.	2:5	90.73 ± 0.5	95 ± 1.05%
10.	1:3	91.2 ± 1.1	61 ± 1.07%
11.	5:2	54.89 ± 3.0	66 ± 1.21%
12.	4:1	53.35 ± 2.9	60 ± 1.63%
13.	1:5	92.91 ± 0.8	82 ± 1.2%
14.	6:1	62.93 ± 3.7	75 ± 0.71%
15.	1:6	92.76 ± 1.6	97 ± 0.94%

Preparation of mPEG-PLGA-RIS-HA Nanoparticles

The nanoparticles with modified mPEG and RIS-HA is prepared by the dialysis method (Fu *et al.*, 2014) (20). In brief, 100 mg RIS-HA–PLGA conjugate and mPEG–PLGA block copolymer were dissolved in 20 mL DMF (dimethylformamide). The solution was dialyzed for 12 h in a dialysis tube (Mol. wt. cut-off: 12 000 Da) in 500 mL of pH-7.4 PBS, which was replaced every 2 h. The final suspension was filtered through a syringe filter with a 0.45 µm pore size. Different ratio of mPEG-PLGA and PLGA-RIS-HA were used. The ratio was optimized on the basis of particle size and entrapment efficiency (Table II). The prepared nanoparticles were further characterized for various *in vitro* and *in-vivo* attributes.

In-vitro Characterization

The prepared mPEG-PLGA-RIS-HA nanoparticles are characterized for following parameters.

Surface Morphology

Morphological characterization of the nanoparticles was done by transmission electron microscopy. Samples of nanoparticles formulations were examined under microscope and photographed at a magnification of 100X, by

Table II Different Ratio for the Formulation Optimization

Sample code	Ratio of polymer (PLGA-mPEG : PLGA-drug–HA)(mg)	Particle size mean ± SD (nm)	PdI	Entrapment efficiency mean ± SD (%)
RISHA 1	1:1	79.69 ± 2.3 nm	0.27 ± 3.4	93 ± 3.1%
RISHA2	1:5	101 ± 4.9 nm	0.36 ± 2.7	68 ± 1.1%
RISHA3	1:3	145 ± 1.0 nm	0.20 ± 1.9	75 ± 2.6%
RISHA4	1:4	115 ± 3.6 nm	0.54 ± 2.3	60 ± 5.6%
RISHA5	1:6	88.26 ± 1.2 nm	0.70 ± 3.0	89 ± 2.8%

means of a fitted camera. Negatively stained samples of nanoparticles were examined by transmission electron microscope at 70 kV.

Size Distribution and Zeta Potential Measurement

Measurement of particle size, zeta potential and polydispersity of nanoparticles was performed by done using Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK), which is based on the principle of dynamic light scattering (DLS). All DLS measurements were done in triplicate at 25°C at a detection angle of 90°. For zeta potential measurements, disposable capillary cell with a capacity of 1 mL was used. To obtain complete dispersion, the nanoparticles were dispersed in Marcol 52 (Exxon Mobil Co., USA) and sonicated for 10 min at 120Wpower (Branson 8210, Branson Ultrasonics Co., Danbury, CT, USA).

In vitro Release Studies

The dialysis method was used to perform the *in-vitro* release of drug from developed nanoparticles. Two milliliters of the nanoparticles suspension was sealed in a dialysis tube (Mol. wt. cut-off: 12 000 Da, flat with 25 mm, a diameter of 16 mm, a capacity of 60 mlft) and dialyzed against 100 mL PBS, incubated with gentle shaking at 37°C. At predetermined time intervals, 1 mL of the sample was collected from the released media and replaced with fresh Phosphate buffer solution (PBS:pH-7.4). The released risedronate(RIS) was analyzed by *UV/VIS* spectrophotometer at 263 nm.

In-vivo Pharmacodynamic Studies

Animals

In-house laboratory bred healthy thirty six Wistar rats of 12 weeks age were used in this study. Animals were preserved under controlled temperature at 25 ± 2°C with 12 h light/dark cycle with food and water provided *ad libitum*. The experiments were directed as per the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines after obtaining permission from Jamia Hamdard (Approval no. 770), Institutional Animal Ethics Committee, New Delhi and were adhered to during complete study period.

Pre-Clinical Study Design

The comparative *in-vivo* pharmacodynamic studies were carried out with 30 male Wistar rats ($n = 3$, weighing around 250 g) divided randomly into 5 groups as following. Osteoporosis was induced in Group 2, 3, 4 and 5 by dexamethasone (0.1 mg/kg) and left for 6 weeks for the development of osteoporosis (23). Groups 3, 4 and 5 were further treated with different formulations for 1 month. The details are given in (Table III).

Volumetric Bone Density Analysis

The left femur of each rats were cleaned of soft tissues and then refrigerated at −20°C. Before the densitometric examination, it was first thawed for 30 min. The femur were examined by densitometer using a Hologic (QDR 4500, USA) after

Table III Study Designing for Pharmacodynamic Analysis

Groups	Name of groups($n = 3$)	Dose	Route of administration
Grp1	Control (Normal Saline)	0.1 mg/kg of weight	Subcutaneous (s.c.)
Grp2	DEX induced Osteoporotic Model (Dexamethasone)	0.1 mg/kg of weight	Subcutaneous(s.c.)
Grp3	mPEG-PLGA-RIS-HA nanoparticles	0.5 mg/kg of weight	Intravenous (i.v.)
Grp4	mPEG-PLGA-RIS nanoparticle	0.5 mg/kg of weight	Intravenous (i.v.)
Grp5	RISOFOS (Marketed Product)	0.5 mg/kg of weight	Oral

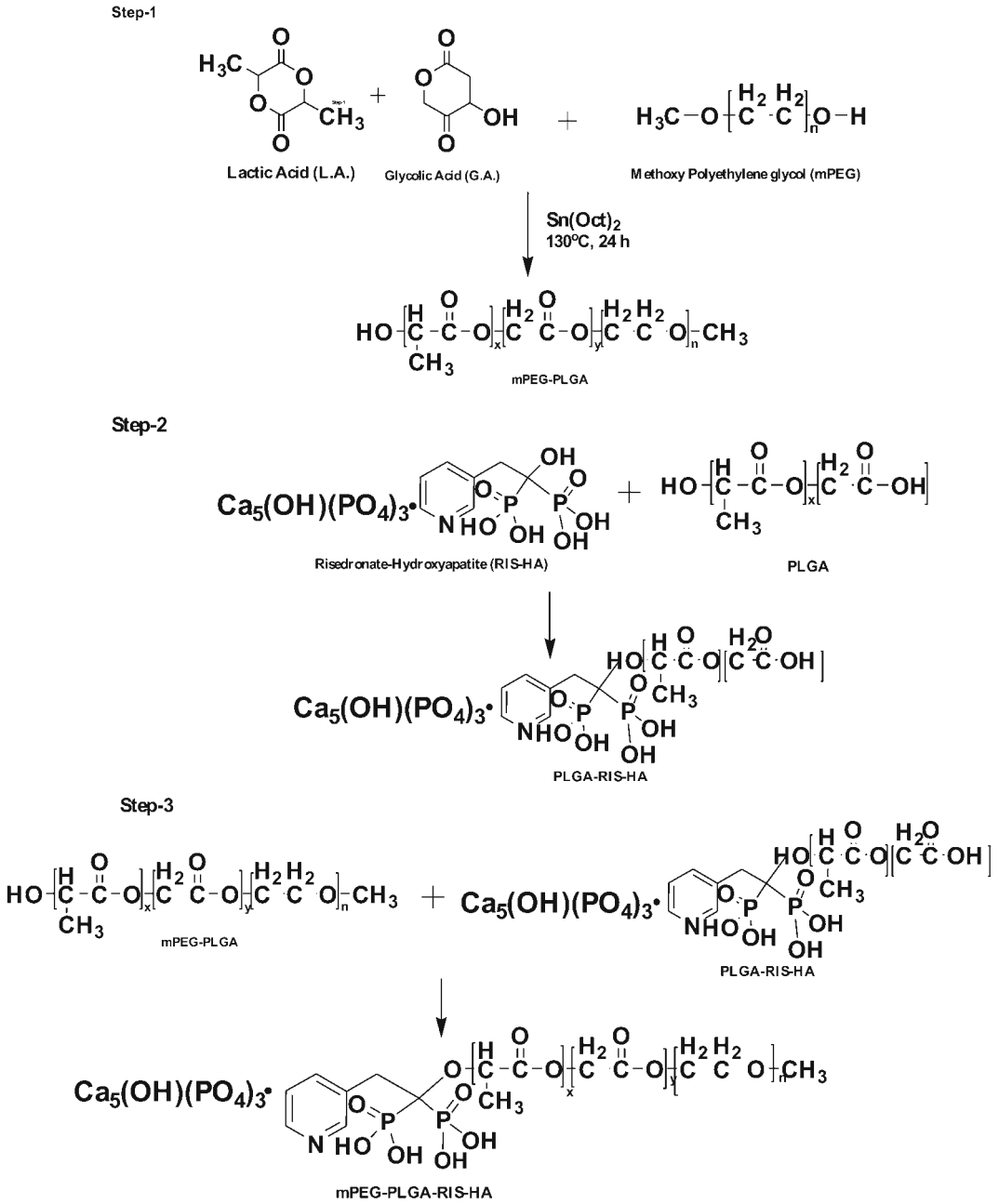

Fig. 1 Synthetic pathway for the preparation of mPEG-PLGA-RIS-HA Nanoparticle.

1 month treatment taken as therapeutic measure of each rat group.

Histopathological Observation

The left femur was fixed in 10% formalin for 12 h at 4°C, decalcified in 5% ethyenediamine tetracetic acid (EDTA) for 7 days and embedded in paraffin wax, cut into sagittal plane section of 5 µm thickness of the femur. The sections were stained with hematoxylin and eosin (HE), and examined for histopathological changes under a light microscope (24).

Statistical Analysis

All data has been stated as the mean ± standard deviation (SD). For all the data, comparisons between different treatments were examined by one-way ANOVA followed by Tukey's multiple comparison tests. In all test, a probability error of less than 0.05 was carefully chosen as the criterion

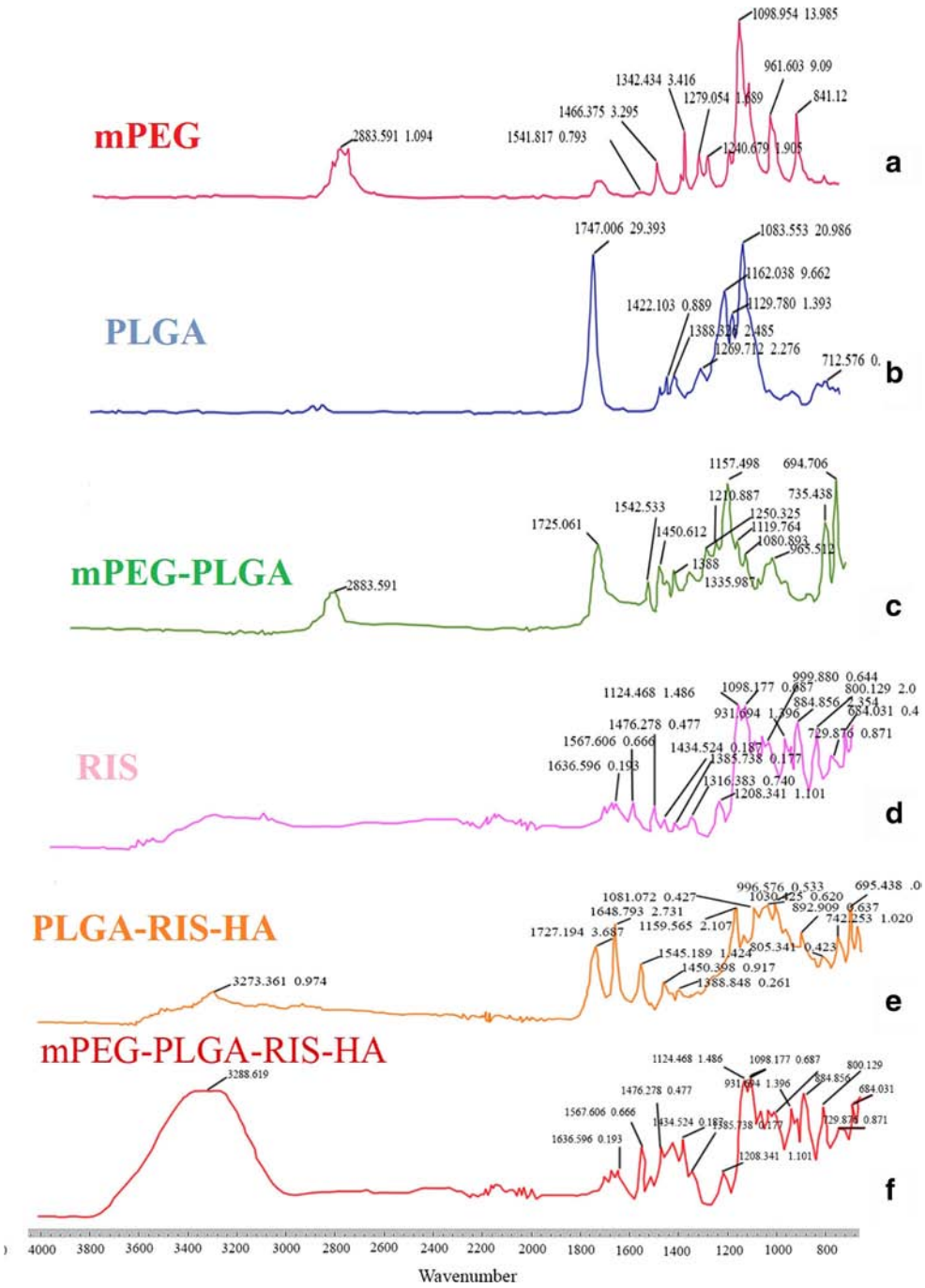

Fig. 2 FTIR of (**a**): mPEG, (**b**): PLGA, (**c**): mPEG-PLGA (**d**): Risedronate, (**e**): PLGA-RIS-HA conjugate and (**f**): mPEG-PLGA-RIS-HA Nanoparticles.

for statistical significance. Bar graphs were drawn using GraphPad Prism and Matlab software.

RESULTS AND DISCUSSION

Schematic representation of overall procedure for the preparation of surface-modified nanoparticles is shown in (Fig. 1). The dialysis method, which does not require surfactant, was selected for the preparation of nanoparticles as the usage of additional surfactant can reduce the functionality of RIS. Both RIS and mPEG were found to exist on the surface of nanoparticles due to the hydrophilicity of those molecules. The mPEG–PLGA conjugate plays a role of a stabilizer and thus additional stabilizers are not needed for the formation of stable nanoparticles.

Preparation and Characterization of Nanoparticle

The synthesis of mPEG–PLGA block copolymers was based on ring opening polymerization as it provides colloidal stability and hydrophilicity to the prepared

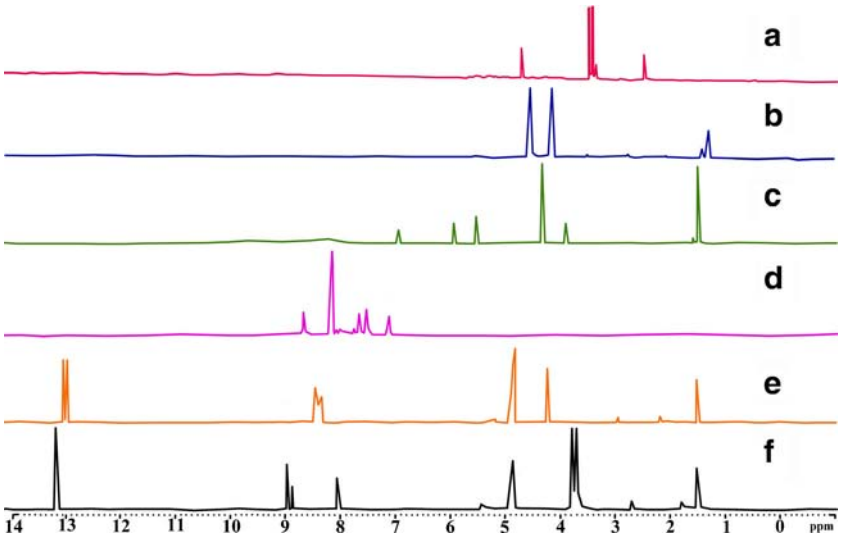

Fig. 3 NMR of (**a**): mPEG, (**b**): PLGA, (**c**): mPEG-PLGA. (**d**): RIS, (**e**): PLGA-RIS-HA and (**f**): mPEG-PLGA-RIS-HA Nanoparticles.

nanoparticles (18). The FT-IR spectra of the mPEG–PLGA copolymer, mPEG, and PLGA are shown in (Fig. 2a-c). The major absorption bands for mPEG–PLGA were observed in the region of 2800–3000 cm^{-1} (C–H stretching of CH_3), 1725 cm^{-1}(ester C=O stretching), and 1080 cm^{-1} (O–CH_2 stretching) respectively. Therfore, the FT-IR study confirmed that the mPEG–PLGA block copolymers were successfully synthesized.

The successful conjugation of mPEG-PLGA was further confirmed by performing ^{1}H NMR study of all components.

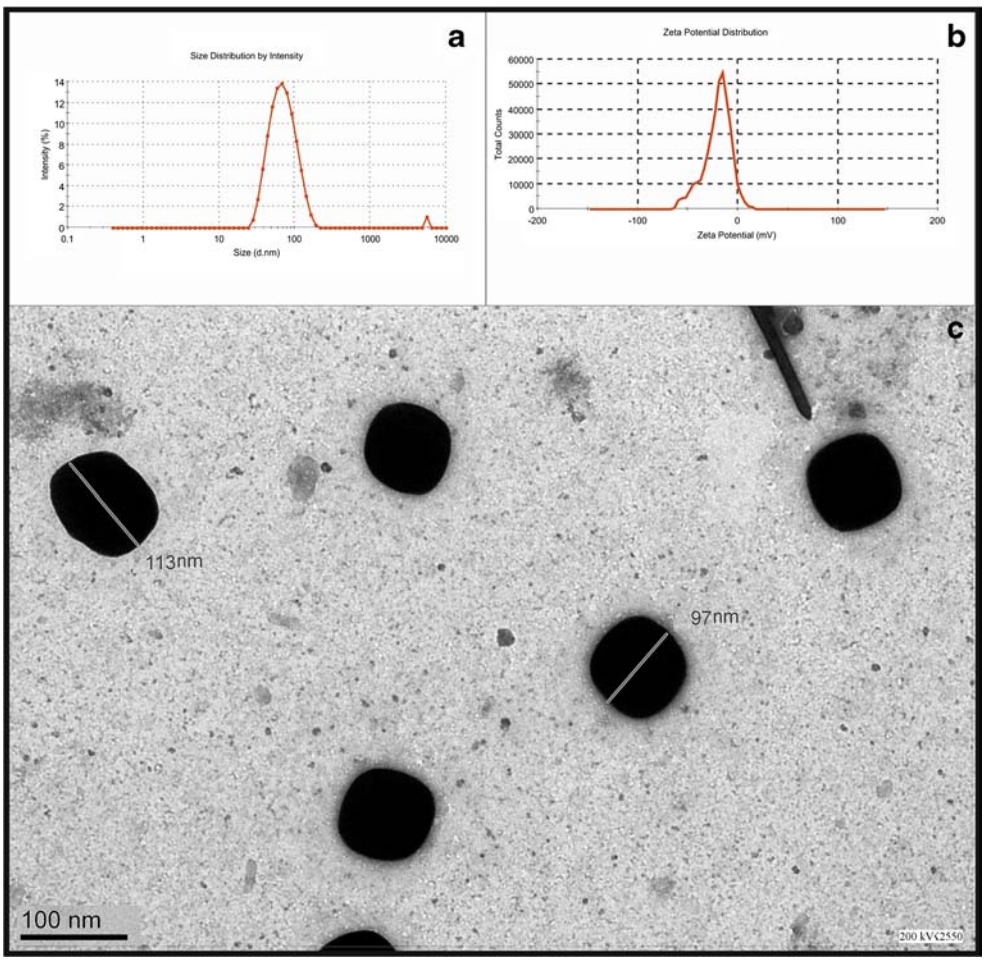

Fig. 4 (**a**) Size distribution, (**b**) surface charge and (**c**) surface morphology of mPEG-PLGA-RIS-HA nanoparticles.

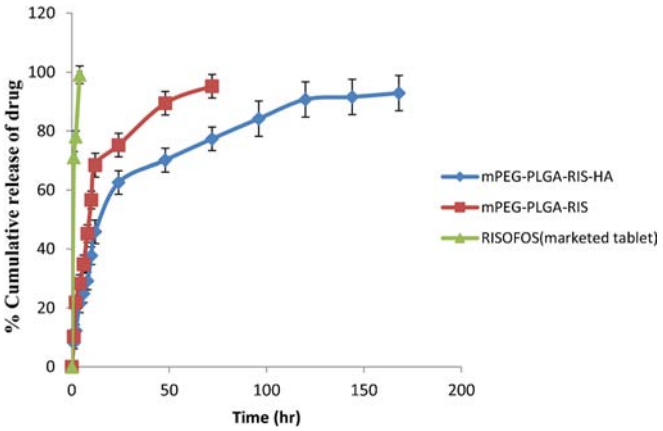

Fig. 5 *In-vitro* cumulative release of mPEG-PLGA-RIS, mPEG-PLGA-RIS-HA nanoparticles and RISOFOS marketed tablet in PBS-7.4.

The large peak at 3.45 ppm was observed for the methylene group of mPEG (Fig. 3a). The multiplets at 4.91 and 4.38 ppm were corresponding to the glycolic acid and peak at 1.55 ppm for methyl groups of lactic acid were observed (Fig. 3b). The ^{1}H NMR of mPEG-PLGA copolymer also showed the peaks in the similar reasons for mPEG and PLGA (Fig. 3c).

For the preparation of mPEG-PLGA-RIS-HA nanoparticles, the DCC and NHS activated PLGA was first conjugated with RIS-HA moiety and then conjugated with mPEG-PLGA copolymer. The FT-IR spectra of RIS-HA-PLGA nanoconjugate exhibited absorption bands at 900–1200 cm^{-1} (overlapping of pyridine ring with PO_3^{-4}) for risedronate, at 3200–3300 cm (1) (stretching of OH), and 1600–2400 cm^{-1}(O=P-H stretching) for hydroxyapatite and at 3273 cm^{-1}(C-O-H stretching) for PLGA (Fig. 2d-e).

^{1}H NMR study of RIS-HA-PLGA revealed, the large peaks at 13 ppm for hydroxyapatite ($CaHPO_4$) (Fig. 3e) and at 8.5 ppm for the RIS (Risedronate sodium).

The FT-IR of final nanoparticles mPEG-PLGA-RIS-HA exhibited absorption band of mPEG-PLGA at 1725 cm^{-1}(ester C=O stretching), and 1080 cm^{-1} (O–CH_2 stretching), 900–1200 cm^{-1} (overlapping of pyridine ring with PO_3^{-4}) for risedronate, at 3200–3800 cm^{-1} (stretching of OH), and 1636–2300 cm^{-1}(O=P-H stretching) for hydroxyapatite (Fig. 2f).

The ^{1}H NMR spectra of PEG-PLGA-RIS-HA Nanoparticles (Fig. 3f) revealed peaks at 1.7 ppm for mPEG, for PLGA containing lactic acid and glycolic acid at 4.9 and 3.8 ppm respectively. On the other hand peak of the methyl groups of lactic acid was observed in same positions at1.5 ppm. Risedronate peaks was observed at 8.0 to 9.12 ppm and for hydroxyapatite peaks was found to be at 13.23 ppm.

The size, zeta-potential and entrapment efficiency of mPEG-PLGA-RIS-HA Nanoparticles were found to be 79.69±2.3 nm, −25±0.34 mV and 93±3.10% respectively. (Fig. 4a-b and Table II). TEM analysis confirmed the uniformity of mPEG-PLGA-RIS-HA nanoparticles (Fig. 4c).

In-vitro Drug Release

In the present study, RIS serves mainly as a targeting moiety to guide the nanoparticles to bone and HA is being a major

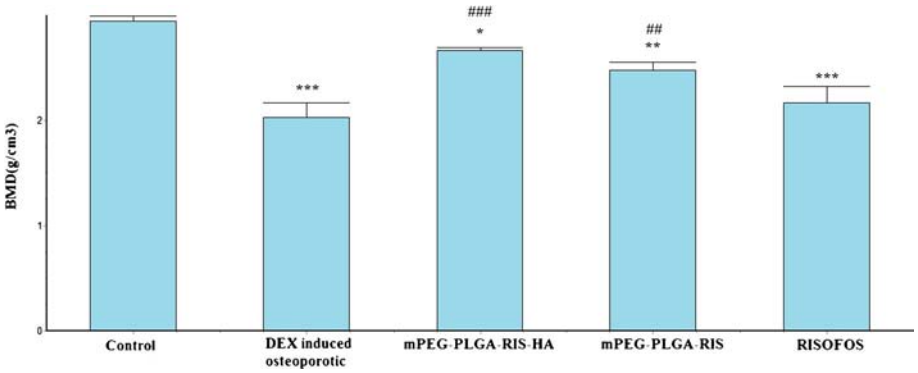

Fig. 6 VMD analysis of osteoporotic induced model after treatment with different formulations. VMD analysis of osteoporotic induced model after treatment with different formulations. Data are shown as the mean ± SD ($n = 3$), evaluated by Tukey's multiple comparison test. ***Significantly statistical comparison with control group ($p < 0.001$), *Significantly statistical comparison with control group ($p < 0.05$), **Significantly statistical comparison with control group ($p < 0.01$), ###Significantly statistical comparison with DEX induced osteoporotic group ($p < 0.001$), ## Significantly statistical comparison with DEX induced osteoporotic group ($p < 0.01$).

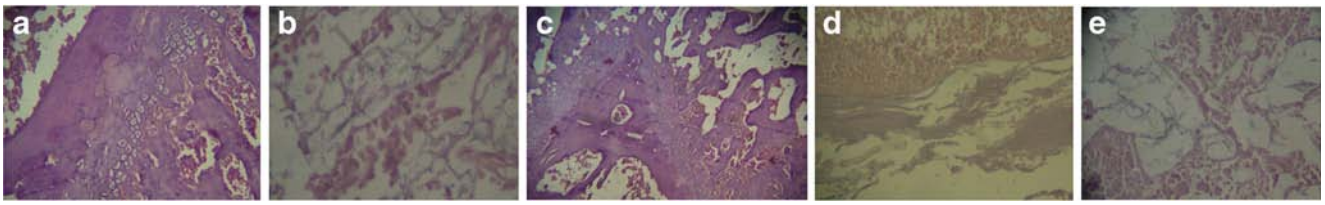

Fig. 7 Histology of femur (**a**–**e**) (100×): (**a**): Control, (**b**): DEX induced osteoporotic model, (**c**): mPEG-PLGA-RIS-HA (i.v.), (**d**): mPEG-PLGA-RIS (i.v.), (**e**): RISOFOS (oral marketed tablet).

component of bone used as a drug, to produce synergistic effect to treat osteoporosis. The prepared mPEG-PLGA-RIS-HA nanoparticle exhibited biphasic release pattern. The early burst in the release of risedronate was observed because the Risedronate was chemically conjugated to the polymer but physically entrapped inside and outside the core of the mPEG-PLGA-RIS-HA nanoparticles. However, the release of RIS from mPEG-PLGA-RIS-HA was found to be 93±3.10% within 168 h and for mPEG–PLGA-RIS nanoparticles without hydroxyapatite it was found to be 95.2±1.9% within 72 h. While the marketed tablet RISOFOS showed 99.1±4.6% release of drug within 2 h. It has been observed that the mPEG-PLGA-RIS-HA nanoparticles provide optimum and controlled release of drug (Fig. 5).

In- vivo Pharmacodynamic Evaluation

Volumetric Bone Density Analysis(VBD)

Volumetric bone density level of the left femur was significantly lowered in DEX group (1.97±0.083 g/cm^3) as compared to the control group (2.57±0.58 g/cm^3) ($p<0.001$). While the VBD of the femur of the rats treated with mPEG-PLGA-RIS-HA nanoparticles was found to be significantly increased (2.52±0.69 g/cm^3) than the VBD of DEX group (1.97±0.083 g/cm^3). On the other hand the VBD of the femur of DEX induced rats treated with mPEG-PLGA-RIS nanoparticles and with RISOFOS(marketed tablet) was found to be 2.43±0.41 and 2.02±0.49 g/cm^3 respectively which was significantly higher when compare to DEX induced group($p<0.01$ and $p<0.05$) respectively. While comparing the results for each therapy as shown (Fig. 6), the mPEG-PLGA-RIS-HA showed a greater increase in VBD then all other treatments. This may be presumably attributed due to the presence of HA in this formulation. The HA is a natural inorganic component of the bone which is further responsible for osteogenesis and bone regeneration (6). In bone tumor surgeries, the filling of HA has been also reported. Healing of lesions upon incorporation of HA with good bone formation has been reported in literature (25). Thus, whether administered prophylactically or therapeutically, bisphosphonates were found to accelerate bone healing. Bisphosphonates selectively disrupt osteoclast activity. Thus, the highly selective localization, bone retention and anti-osteoclastic property make the bisphosphonates, the drug of choice in the present scenario (26, 27).

Histopathological Evaluation

The result of histopathological evaluation of femur from each group of rats is shown in Fig. 7. Normal dense micro-architecture of the diaphysis and compact trabeculae was observed in control group (Fig. 7a). The femur of osteoporotic (DEX-induced) group rats showed severe thinning of the trabeculae, disappearance of growth plate and widened inter-trabecular spaces due to loss of connectivity among the bone tissues (Fig. 7b). In contrast to this histology of bone treated with formulation- mPEG-PLGA-RIS-HA nanoparticles showed a restoration of architecture of trabecular bone with well connected bone matrix and narrow inter-trabecular spaces (Fig. 7c). Comparatively slight improvement was observed on the ruffled border as well as in Harvesian system and bone matrix when treated with mPEG-PLGA-RIS (Fig. 7d). No significant changes in bone architecture of femur of the rats were observed when treated with oral marketed tablet(RISOFOS). The maximum restoration (appearance of dense bone matrix, thick ruffled border) was observed in femur treated with mPEG-PLGA-RIS-HA (Fig. 7c). The results are very well corroborated with the VBD results. This may be attributable to the synergistic effect of bisphosphonate and hydroxyapatite. This approach can be used as a novel therapy for the treatment of osteoporosis with high selectivity for bone tissues as well as reduce the risk of side effects associated with bisphosphonates (6, 19).

CONCLUSION

The outcomes from the present study lead us to a clear indication that as compared to existing RIS drug therapy, the developed m-PEG-PLGA-RIS-HA nanoparticles are highly effective in promoting the formation of bone in DEX induced osteoporotic rat model. In addition, mPEG-PLGA-RIS-HA nanoparticles is a novel drug therapy that is most likely to benefit postmenopausal women suffering from osteoporosis as well as senile osteoporosis and is proved to have better therapeutic outcomes for the effective management of osteoporosis with high selectivity for bone tissues.

Pharmacodynamic studies clearly indicated that the osteoclast inhibition function of developed nanoparticles was attributed to bone density analysis as well as histopathological evaluation. Thus, mPEG-PLGA-RIS-HA nanoparticles with hydroxyapatite can provide an effective solution for osteoclast inhibition with targeted specific drug delivery.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors are thankful for the financial assistance from ICMR, New Delhi. We also acknowledge the Evonik Research for providing the gift sample of PLGA (50:50) polymer and lipoid for providing the gift sample of methoxy PEG. The authors are also thankful to Mr. Jitender singh, Department of Pharmaceutical chemistry Jamia Hamdard and Dr. Ambrish Tiwari, Incharge of animal house, Faculty of Science, Jamia Hamdard, New Delhi for their inputs regarding animal studies.

Conflicts of Interest The authors state no conflicts of interest

REFERENCES

1. Kristina A. Bull World Health Organ. 2003;81:657–63.
2. Cooper C, Melton LJ. Epidemiology of osteoporosis. Trends Endocrinol Metab. 1992;3:224–9.
3. Nordin BEC. International patterns of osteoporosis. Clin Orthop. 1966;45:17–30.
4. Currey JD. Biomechanics of mineralized skeletons. In: Carter JG, editor. Skeletal biomineralization: patterns, processes and evolutionary trends, I. New York: Van Nostrand Reinhold; 1990.
5. Lin L, Chow KL, Leng Y. Study of hydroxyapatite osteo inductivity with an osteogenic 257 differentiation of mesenchymal stem cells. J Biomed Mater Res A. 2009;89:326–35.
6. Negi P, Negi LM, Vohora D, Ahmad FJ, Talegaonkar S. Enhancing safety and efficacy of bisphosphonate therapy by association with hydroxyapatite as adjuvant drug carriers. OA Med Hypothesis. 2014;2:6.
7. Bonnick S, Saag KG, Kiel DE. Comparison of weekly treatment of postmenopausal osteoporosis with alendronate vs. risedronate over two years. J Clin Endocrinol Metab. 2006;91:2631–7.
8. Kathryn L, Kavanagh KG, James ED, Xiaoqiu W, Stefan K, Ebetin FH, *et al*. The molecular mechanism of nitrogen-containing bisphosphonates as anti-osteoporosis drugs. Proc Natl Acad Sci U S A. 2006;103:7829–34.
9. Vaculikova E, Placha D, Pisarcik M, Peikertova P, Dedkova K, Devinsky F, *et al*. Preparation of Risedronate nanoparticles by solvent evaporation technique. Molecules. 2014;19:17848–61.
10. Nasr M, Awad GA, Mansour S, Taha I, Al Shamy A, Mortada ND. Different modalities of NaCl osmogen in biodegradable microspheres for bone deposition of risedronate sodium by alveolar targeting. Eur J Pharm Biopharm. 2011;79:601–11.
11. Cenni E, Granchi D, Avnet S, Fotia C, Salerno M, Micieli D, *et al*. Biocompatibility of poly(D, L-lactide-co-glycolide) nanoparticles conjugated with alendronate. Biomaterials. 2008;29:1400–11.
12. Bigi A, Boanini E, Capuccini C, Fini M, Mihailescu IN, Ristoscu C, *et al*. Biofunctional alendronate–Hydroxyapatite thin films deposited by matrix assisted pulsed laser evaporation. Biomaterials. 2009;30:6168–77.
13. Hruby M, Konak C, Ulbrich K. Polymeric micellar pH sensitive drug delivery system for doxorubicin. J Control Release. 2005;103:137–48.
14. Shi X, Wang Y, Varshney RR, Ren L, Zhang F, Wang DA. In-vitro osteogenesis of synovium stem cells induced by controlled release of bisphosphonate additives from microspherical mesoporous silica composite. Biomaterials. 2009;30:3996–4005.
15. Niemi R, Vepsalainen J, Taipale H, Jairvinen T. Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel acyloxyalkyl esters of clodronic acid. J Med Chem. 1999;2:5053–8.
16. Ezra A, Hoffman A, Breuer E, Alferiev IS, Monkkonen J, El Hanany-Rozen N, *et al*. A peptide prodrug approach for improving bisphosphonate oral absorption. J Med Chem. 2000;43:3641–52.
17. Ogawa K, Mukai T, Inoue Y, Ono M, Saji H. Development of a novel 99mTc-chelate conjugated bisphosphonate with high affinity for bone as a bone scintigraphic agent. J Nucl Med. 2006;47:2042–7.
18. Choi SW, Kim JH. Design of surface-modified poly(D, L-lactide-co-glycolide) nanoparticles for targeted drug delivery to bone. J Control Release. 2007;122:24–30.
19. Sahana H, Khajuria DK, Razdan R, Mahapatra DR, Bhat MR, Suresh S, *et al*. Improvement in bone properties by using risedronate adsorbed hydroxyapatite novel nanoparticle based formulation in a rat model of osteoporosis. J Biomed Nanotechnol. 2013;9:193–201.
20. Fu YC, Fu TF, Wang HJ, Lin CW, Lee GH, Wu SC, *et al*. Aspartic acid-based modified PLGA-PEG nanoparticles for bone targeting: in vitro and in vivo evaluation. Acta Biomater. 2014;10:4583–96.
21. Wu CC, Wang CC, Lu DH, Hsu LH, Yang KC, Lin FH. Calcium phosphate cement delivering zoledronate decreases bone turnover rate and restores bone architecture in ovariectomized rats. Biomed Mater. 2012;7:035009.
22. Kyllonen L, Este MD, Alini M, Eglin D. Local drug delivery for enhancing fracture healing in osteoporotic bone. Acta Biomater. 2015;11:412–34.
23. Bozzo Rde O, Rocha RG, Haiter Neto F, Paganini GA, Cavalcanti MG. Linear density analysis of bone repair in rats using digital direct radiograph. J Appl Oral Sci. 2004;12:317–21.
24. Bancroft JD, Cook HC. Manual of histological techniques and their diagnostic application. Edinburgh: Churchill Livingstone; 1994.
25. Saikia KC, Bhattacharya TD, Bhuyan SK, Talukdar DJ, Saikia SP, Jitesh P. Calcium phosphate ceramics as bone graft substitutes in filling bone tumor defects. Indian J Orthop. 2008;42:169–72.
26. Murakami H, Takahashi N, Sasaki T, Udagawa N, Tanaka S, Nakamura I, *et al*. A possible mechanism of the specific action of bisphosphonates on osteoclasts: tiludronate preferentially affects polarized osteoclasts having ruffled borders. Bone. 1995;17:137–44.
27. Khanna S, Pillai KK, Vohora D. Bisphosphonates in phenytoin-induced bone disorder. Bone. 2011;48:597–606.