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4 **Star-shaped poly(oligoethylene glycol) copolymer-based gels: thermo-**  
5 **responsive behaviour and bioapplicability for risedronate intranasal delivery**

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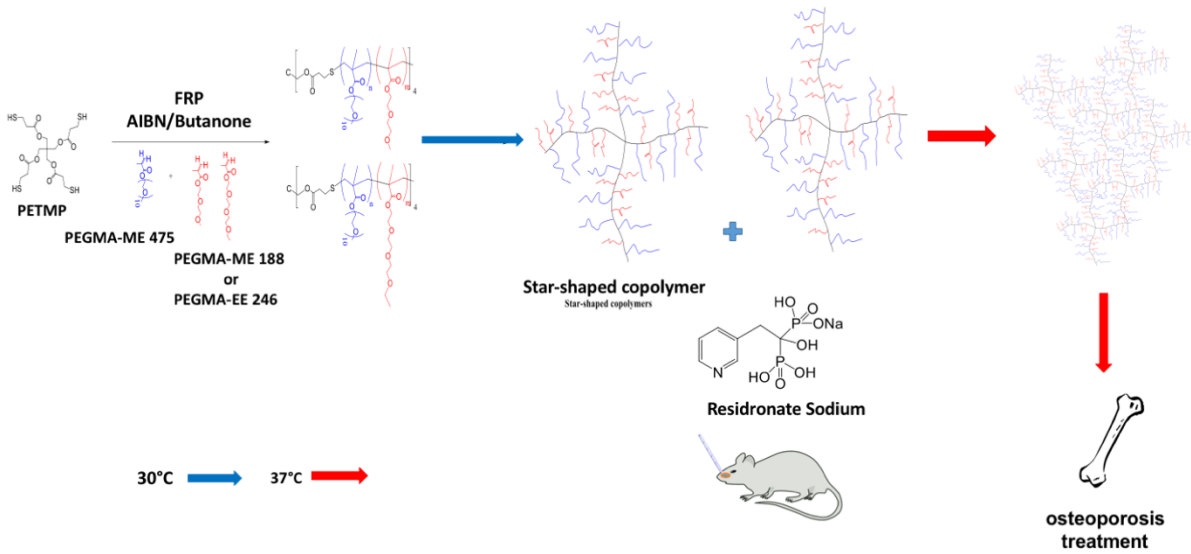
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32 **Graphical Abstract**



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54 **Abstract**

55 The aim of this work was to obtain an intranasal delivery system with improved mechanical and  
56 mucoadhesive properties that could provide prolonged retention time for the delivery of  
57 risedronate (RS). For this, novel in situ forming gels comprising thermo-responsive star-shaped  
58 polymers, utilizing either polyethylene glycol methyl ether (PEGMA-ME 188, Mn 188) or  
59 polyethylene glycol ethyl ether (PEGMA-EE 246, Mn 246), with polyethylene glycol methyl ether  
60 (PEGMA-ME 475, Mn 475), were synthesized and characterized. RS was trapped in the selected  
61 gel-forming solutions at a concentration of 0.2% w/v. The pH, rheological properties, in vitro drug  
62 release, ex vivo permeation as well as mucoadhesion were also examined. MTT assays were  
63 conducted to verify nasal tolerability of the developed formulations. Initial in vivo studies were  
64 carried out to evaluate anti-osteoporotic activity in a glucocorticoid induced osteoporosis model  
65 in rats. The results showed successful development of thermo-sensitive formulations with  
66 favorable mechanical properties at 37°C, which formed non-irritant, mucoadhesive porous  
67 networks, facilitating nasal RS delivery. Moreover, sustained release of RS, augmented  
68 permeability and marked anti-osteoporotic efficacy as compared to intranasal (IN) and intravenous  
69 (IV) RS solutions were realized. The combined results show that the in-situ gels should have  
70 promising application as nasal drug delivery systems.

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77 **Keywords:**

78 Nasal delivery; thermo-responsive star-shaped polymers; risedronate; thermogel;  
79 cytocompatibility; antiosteoporotic activity.

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## 81        **1. Introduction**

82        Star-shaped polymers are formed of a core connected to three or more linear chains (arms) with  
83        similar lengths (Daoud and Cotton, 1982). They not only have condensed structure with small  
84        volume and size compared to linear analogues but also can be decorated with different end groups  
85        that make them beneficial in different applications (Dong et al., 2015). Arms of star-shaped  
86        polymers may be either homo-, or co-polymers, hence the final properties of the resulting polymers  
87        (e.g., star-block and heterostar copolymers) may be adjusted by choosing the respective chemical  
88        structure of an arm and core, depending on the required application (Aloorkar et al., 2012).

89        Among star-shaped polymers, polyethylene oxide (PEO) based polymers have been prepared,  
90        tested and characterized for many biomedical, pharmaceutical and tissue engineering applications  
91        (Harris, 2013). PEO based star-shaped polymers are non-ionic and biocompatible macromolecules  
92        that have high stability and solubility, hence can function to shield drug payloads from inactivation  
93        by the immune system (Lapienis, 2009). Moreover, designing polymers to have PEO chains  
94        incorporated in star architectures may be advantageous compared to linear PEO counterparts as  
95        these materials can have multiple functional groups at chain-ends or in core-components, adding  
96        to the applicability of these polymers (Gasteier et al., 2007).

97        Recently, star-shaped PEO-based polymers have been tested for their thermoresponsive properties  
98        (Badi and Lutz, 2009). These polymers were found to have a defined lower critical solution  
99        temperature (LCST) in both water and physiological fluids. The thermoresponsive behaviour of  
100        these polymers is thought to be due to their amphiphilic nature (hydrophilic oligo(ethylene glycol)  
101        side chains and hydrophobic backbones) (Jeong et al., 2012).

102        The copolymerization of two oligo(ethylene glycol) methacrylates with different chain lengths,  
103        namely PEGMA-ME 188 and PEGMA-ME 475 was reported and produced thermoresponsive  
104        copolymers with adjustable phase transition temperature values (Saeed et al., 2011). The reversible  
105        phase changes of these copolymers were found to be affected by their concentration and chain  
106        length as well as ionic strength (Magnusson et al., 2008). Hence, these polymers were suggested  
107        as promising candidates for the formulation of thermoresponsive materials. Oligo(ethylene  
108        glycol)-based thermoresponsive materials have now been developed in the form of dendrimers,  
109        microgels, and added to silica particles, gold particles, block copolymer aggregates, carbon  
110        nanotubes, and planar surfaces (Lutz, 2011).

111 Hydrogels were used for different applications in drug and cell delivery as well as in tissue  
112 engineering as polymer scaffold (Cespi et al., 2014; Caló and Khutoryanskiy, 2015). In situ gelling  
113 hydrogels have been exploited for drug delivery through various administration routes and  
114 presented a great interest for nasal delivery (Cai et al., 2011; Galgatte et al., 2014; Wavikar et al.,  
115 2017).

116 Osteoporosis and Paget's disease of bone are major problems in women and geriatric patients  
117 (Riggs and Melton, 1995). In this regard, various pharmacological molecules have been attempted  
118 to accelerate new bone formation such as recombinant human fibroblast growth factor-2,  
119 thrombin-related peptide, bone morphogenetic protein-2 and bisphosphonates (Hirabayashi and  
120 Fujisaki, 2003). Bisphosphonates, as pyrophosphate analogues, possesses a known affinity for  
121 bone tissue and an outstanding antiresorptive and osteoclast inhibitory potential (Czuryszkiewicz  
122 et al., 2005). These types of drugs are effective in the treatment of Paget's disease, osteoporosis,  
123 multiple myeloma, bone metastases, fibrous dysplasia of bone, and breast cancer (Czuryszkiewicz  
124 et al., 2005; Nancollas et al., 2006). Nitrogen containing bisphosphonates (e.g. Risedronate (RS))  
125 are many times more potent than the non-nitrogen ones. Despite the interest in fabricating RS  
126 carrier platforms, its high polarity and hydrophilicity lead to very poor oral bioavailability (< 1%).  
127 In addition, RS exacerbates serious side effects in the gastrointestinal tract (GIT) such as  
128 oesophagitis, ulcers and gastritis. Various administration routes for RS, including intravenous,  
129 subcutaneous and intramuscular routes, have been attempted in order to improve its poor oral  
130 bioavailability (< 1%) and therapeutic efficacy as well as minimizing its side effects (Salzano et  
131 al., 2011). Following intravenous (IV) administration, calcium complexes in the blood are formed  
132 resulting in possible occurrence of renal failure (Toussaint et al., 2009). Furthermore, injection of  
133 sodium salts was reported to develop pain and tissue necrosis at the injection site. Accordingly, a  
134 focus of current research is localized delivery of RS via nasal and pulmonary routes (Fazil et al.,  
135 2016; Nasr et al., 2011).

136 In the present work, polymers of star-shaped PEGMA-ME 188 or PEGMA-EE 246 with and  
137 without PEGMA-ME 475 were synthesized and characterized for preparing thermoresponsive  
138 hydrogels. We aimed to investigate the utilization of the prepared platforms with PEGMA-ME  
139 188 or the PEGMA-EE 246 -co- PEGMA-ME 475 as bio-applicable thermogels with optimal  
140 thermoresponsive and mucoadhesive properties in physiological conditions for the nasal delivery  
141 of RS. To our knowledge, this is the first study to demonstrate star-shaped poly(oligoethylene

142 glycol) copolymers in situ gels as nasal delivery carriers for RS and to evaluate their *in vivo*  
143 efficacy for the treatment of osteoporosis in rat models.

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## 146 **2. Experimental**

### 147 **2.1. Materials**

148 All solvents and reagents were of analytical or HPLC grade and purchased from Sigma Aldrich  
149 (UK) or Fisher Scientific (UK) unless otherwise stated. Deuterated solvents were from Sigma  
150 Aldrich (UK) or Cambridge Isotopes (UK). Monomers used in this study (Polyethylene glycol  
151 methyl ether (PEGMA-ME 188, Mn 188), polyethylene glycol ethyl ether (PEGMA-EE 246, Mn  
152 246), and polyethylene glycol methyl ether (PEGMA-ME475, Mn 475) were purchased from  
153 Sigma Aldrich (UK) and purified before use by passing through a column filled with neutral  
154 alumina. 2,2- azobisisobutyronitrile (AIBN) was purchased from Sigma Aldrich (UK) then  
155 recrystallized from methanol. Pentaerythritol Tetra (3-mercaptopropionate) (PETMP) and 2-  
156 butanone were used as received from Sigma Aldrich (UK). Risedronate sodium was kindly  
157 supplied by SPIC Pharma (India). Spectra/Por dialysis membrane, 12,000–14,000 molecular  
158 weight cutoff, was purchased from Spectrum Laboratories (Canada). MTT (3-(4,5-  
159 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), HEPES buffer, gentamycin,  
160 dimethylsulfoxide and sodium lauryl sulphate (SLS) from Sigma-Aldrich (UK). Foetal calf serum,  
161 Minimum Essential Medium (MEM), from Lonza (Belgium); and L-glutamine GIBCO®, were  
162 purchased from Thermo Fisher Scientific (USA).

163

### 164 **2.2 Polymer synthesis and telomerization kinetics**

165 Polymers were prepared by free radical polymerization (Fig. S1). Monomers were weighed into a  
166 round bottom flask then PETMP and 20 ml of 2-butanone were added. Lastly AIBN was added  
167 and the mixture was degassed with nitrogen for 25 minutes. Then the solution temperature was  
168 elevated to 70 °C and polymerization was allowed to proceed at that temperature for 2 h. The  
169 reaction was then stopped by cooling the reaction vessel in liquid nitrogen. The polymer was  
170 purified from excess monomers and reagents by precipitation three times in hexane.

171 Telomerization kinetics of Poly(PEGMA188-ME) and Poly(PEGMA246-EE) were examined by  
172 following the concentrations of monomers and telogen (PETMP) throughout the reaction. Samples  
173 were collected at 0, 30, 45, 60, 90, 120, 150 and 180 min and the reaction was stopped by cooling  
174 them in ice.

175 Samples were evaluated by  $^1\text{H-NMR}$  in  $\text{CDCl}_3$  to detect monomer PEGMA188 for  
176 Poly(PEGMA188-ME) and PEGMA246-EE for Poly(PEGMA246-EE) conversion by comparing  
177 the integration of monomer vinylic protons to polymer methine protons. The remaining sample  
178 was precipitated in hexane then filtered and the filtrate was evaporated under vacuum. The residue  
179 left after evaporation was dissolved in water then any PETMP left was determined by iodine  
180 titration to determine telogen conversion (Loubat and Boutevin, 2001).

181

## 182 **2.3 Characterization of the synthesized polymers**

### 183 **2.3.1 FTIR characterization**

184 FTIR spectra of the polymers were recorded in the range of  $4000\text{-}400\text{ cm}^{-1}$  on a Nicolet 6700 FTIR  
185 (Thermo Scientific, USA), equipped with an attenuated total reflectance (ATR) accessory  
186 providing an analysis of the sample surface. Polymeric samples were loaded on KBr discs without  
187 previous treatments or special preparation techniques. All spectra were recorded at ambient  
188 temperature under vacuum to remove air humidity contribution at a resolution of  $4\text{ cm}^{-1}$  and 16  
189 scans were recorded for each measurement to obtain an adequate signal to-noise ratio.

190

### 191 **2.3.2 Nuclear Magnetic Resonance Analysis ( $^1\text{H-NMR}$ )**

192  $^1\text{H-NMR}$  spectra were recorded on a Bruker 400 spectrometer (Bruker Avance III, Switzerland) at  
193  $399.8\text{ MHz}$  ( $^1\text{H}$ ) in chloroform-d solutions. All chemical shifts are reported in ppm relative to  
194 TMS.

195

### 196 **2.3.3. Gel Permeation Chromatography (GPC)**

197 As shown in Table 1, the number average molecular weight ( $M_n$ ), weight average molecular weight  
198 ( $M_w$ ) and polydispersity index ( $M_w/M_n$ ) of the synthesised polymers were determined using gel

199 permeation chromatography device (PL-50 PolymerLabs, UK) equipped with refractive index  
200 detector. The device columns (30 cm PLgel Mixed- C, 2 in series) were eluted with chloroform  
201 and calibrated with polystyrene standards ( $M_w=162\text{--}371100\text{Da}$ ) (PolymerLabs, UK).

202 Standard calibrations as well as analysed samples were maintained at  $40^\circ\text{C}$  during analysis and a  
203 flow rate of eluent was set at  $1\text{ml}/\text{min}$  for all experiments. All samples were dissolved in HPLC  
204 grade chloroform and filtered using  $0.2\ \mu\text{m}$  filter before injecting  $100\ \mu\text{L}$  aliquots.

205

#### 206 **2.3.4. Rheometrical characterizations**

207 The rheological measurements were performed using Physica Rheometer (Anton Paar, USA),  
208 equipped with parallel plate geometry ( $25\ \text{mm}$  diameter) and the gap distance between two plates  
209 was set as  $0.5\text{mm}$ . RheoPlus Software (Version 3.6x) was used for measuring data.

210 Temperature Ramp experiments were recorded at  $0.05\%$  strain at a frequency =  $1\ \text{rad s}^{-1}$  with a  
211 heating rate of  $1^\circ\text{C}/\text{min}$  from  $10$  to  $40^\circ\text{C}$ . Before running the experiment, the lower plate and the  
212 PP25 were left in contact for  $5\ \text{min}$  at  $10^\circ\text{C}$ . Afterwards,  $300\ \mu\text{l}$  of sample was applied on the lower  
213 plate. The sample was equilibrated at the desired temperature ( $10^\circ\text{C}$ ) for  $4\ \text{min}$  before starting the  
214 run. The tests have been performed using a fresh aliquot of sample.

215

#### 216 **2.3.5. UV/ Visible spectroscopy (Temperature dependent turbidity) measurement**

217 A DU 800 UV spectrophotometer (Beckman Coulter, USA) with a thermostat was used for  
218 measuring the change in absorption of polymeric sample solutions over a temperature range of  $10\text{--}$   
219  $50\ ^\circ\text{C}$  at a wavelength of  $550.0\ \text{nm}$ . The temperature was controlled and measured using a Peltier  
220 plate heating system (Beckman Coulter, USA) and was increased at a rate of  $1\ ^\circ\text{C}/\text{min}$ .

221

#### 222 **2.4. Scanning Electron Microscopy (SEM)**

223 Gel surface morphologies were imaged via SEM. Samples were prepared by spreading freeze dried  
224 gels (Poly(PEGMA188-ME-co-PEGMA475-ME) and Poly(PEGMA246-EE-co-PEGMA475-  
225 ME)) on an aluminium stub coated with carbon tape. Samples were then coated with a thin layer  
226 of gold (Blazer SCD 030 Sputter coater, Blazer Union Ltd, Liechtenstein) for  $3\ \text{minutes}$ , then  
227 images were recorded using a JEOL 6060V scanning electron microscope (JEOL Ltd, UK) at an  
228 accelerating voltage of  $30\text{kV}$ .



229

## 230 **2.5. Incorporation of RS in thermoresponsive gels and pH determination**

231 All the co-polymers used in this study were prepared in cold ultrapure water (15-30% w/w), gels  
232 for nasal delivery were prepared to contain 5% mannitol as a tonicity adjuster. The solutions were  
233 kept in a refrigerator for at least 24 h to ensure complete dissolution and formation of clear  
234 homogeneous solutions. The model hydrophilic drug, RS, at a concentration of 0.2% w/v was then  
235 incorporated in the prepared solutions by simple mixing.

236 The developed formulations were evaluated for pH by using a pH meter (Model 3510, Jenway,  
237 UK).

238

239

## 240 **2.6. Viscosity determination of RS loaded thermoresponsive gels**

241 The sol–gel transition behavior of the hydrogels was further evaluated by viscosity measurements.  
242 The viscosity of the instilled formulation into the nose is considered to be very important for  
243 determination of nasal residence times of drugs. The prepared formulations were allowed to gel at  
244 physiological temperature and then the viscosity was determined by a DV-E Viscometer  
245 (Brookfield Engineering Laboratories, USA) using spindle no 52 at speeds ranging from 0.5 to  
246 100 rpm (shear rates 1 to 200 sec<sup>-1</sup>) and over 10% torque. By plotting rheograms of viscosity values  
247 versus shear rate, the flow pattern was checked.

248

## 249 **2.7 Bioadhesion Strength of RS loaded thermoresponsive gels**

250 To quantify mucin-polymer mucoadhesive strength of gel formulation, a simple viscometric  
251 method was used as previously described by (Hassan and Gallo, 1990). The viscosities of 15%  
252 (w/v) porcine gastric mucin dispersions in normal saline, co-polymer solutions and mucin–  
253 hydrogels (RS loaded Poly(PEGMA188-ME-co-PEGMA475-ME) gel (F2) and RS loaded  
254 Poly(PEGMA246-EE-co-PEGMA475-ME) gel (F4)) mixtures were measured at 37 °C using  
255 Brookfield viscometer.

256 The viscosity coefficient was then determined by equation 1:

257

$$\eta_t = \eta_m + \eta_p + \eta_b \quad (1)$$

258 Where  $\eta_t$  is the viscosity coefficient of the system,  $\eta_m$  and  $\eta_p$  are the individual viscosity  
259 coefficients of mucin and polymers, respectively, and  $\eta_b$  is the viscosity component due to the  
260 mucoadhesion.

261

## 262 **2.8 In vitro drug release study**

263 The freshly prepared RS loaded hydrogels were used in in vitro release study similar to the one  
264 attempted by (Nasr et al., 2013). In details, hydrogels equivalent to 2 mg RS were loaded in  
265 cylinders of cellulose acetate membrane and placed in 15 mL vials containing 7.5 ml physiological  
266 normal saline (Wu et al., 2007) maintained at 37 °C in shaker water bath (Kottermann GmbH,  
267 Uetze/Hanigsen, Germany) at 50 rpm. As a control, the release of 2 mg RS solution was also  
268 performed. At selected time intervals (1, 2, 4, 6, 8, 24, and 48 h), 0.5 mL was sampled from vials  
269 and replaced with an equal volume of fresh pre-warmed saline.

270 The quantity of RS released was determined using UV spectrophotometry (UV-Vis  
271 spectrophotometer, UV-1601PC, Shimadzu, Japan) at 262 nm. The results of the release  
272 experiments are shown as cumulative drug percentage released plotted as a function of time.

273 The release profiles was compared using similarity factor ( $f_2$ ) according to (Moore, 1996)  
274 independent mathematical approach The similarity factor ( $f_2$ ) was calculated according to equation  
275 2:

$$276 \quad f_2 = 50 \log \left\{ \left[ 1 + \left( \frac{1}{n} \right) \sum_{t=1}^n (Rt - Tt)^2 \right]^{-0.5} \times 100 \right\} \quad (2)$$

277

278 where n is the number of time points, Rt and Tt are the percent drug released from F2 and F4 at  
279 time t.  $f_2$  value between 50 and 100 indicate that the release profiles are similar.

280

## 281 **2.9 Ex vivo permeation**

282 An ex-vivo nasal permeation study was performed in order to evaluate nasal absorption of RS from  
283 the prepared hydrogels. The study was performed by introducing 100  $\mu$ L of the prepared  
284 formulations in the donor compartment of a Franz-type static glass diffusion cell (Variomag  
285 Telesystem, H + P Labortechnik, Germany) with a 7.5 mL receptor volume and diffusion surface  
286 area: 1.77 cm<sup>2</sup>. The hydrogels were placed on a freshly separated sheep nasal mucosa, positioned  
287 between the donor and receptor compartments. The receptor compartment was filled with  
288 physiological saline (0.9 %, w/w), maintained at 37  $\pm$  0.5 °C. Samples (0.5 ml) were withdrawn

289 from the receptor fluid at appropriate time intervals up to 48 h, and replaced with 0.5 ml of fresh  
290 saline. The results of permeation studies are represented as cumulative drug amount ( $\mu\text{g}$ )  
291 permeated per unit of surface area ( $\text{cm}^2$ ) versus time. The steady state flux ( $J$ ,  $\mu\text{g}/\text{cm}^2/\text{h}$ ) was  
292 determined from the slope of the linear profiles.

293

## 294 **2.10 In vivo study**

295 In the current study, we tried to evaluate role of RS in osteoporosis model induced by  
296 dexamethasone sodium phosphate. All study procedures were evaluated and approved by the  
297 Research Ethics Committee of the Faculty of Pharmacy, Ain Shams University. A total of 24  
298 female Albino rats weighing 225 to 250 g were selected for this study. Animals were housed under  
299 controlled environmental conditions and were maintained in plastic cages with free access to food  
300 and water and were kept at a constant temperature of 20-24°C with 12 h light/dark cycle.

301

### 302 **2.10.1 Glucocorticoid induced osteoporosis model in rats**

303 Following 7 days of acclimatization, glucocorticoid-induced osteoporosis (GIO) was induced in  
304 female Albino rats by subcutaneous (SC) administration of Fortecortin<sup>®</sup> (dexamethasone sodium  
305 phosphate) at 8 mg/kg body weight per 7 days for 4 weeks (Fazil et al., 2016). Weights of rats  
306 were observed during induction of osteoporosis and their treatment.

307

### 308 **2.10.2 Animal groups**

309 The rats were divided into six groups (each group contained 4 rats) as follows:  
310 Group I (S): Received 30  $\mu\text{l}$  intranasal (IN) normal saline (S) through micropipette attached to a  
311 polyethylene tube (negative control group). Group II (DEX): was given subcutaneous (SC)  
312 dexamethasone sodium phosphate (DEX) by above mentioned dose (toxic control group). Group  
313 III (DEX+ IN RS): was given DEX plus receiving IN RS solution (1mg/kg) (Fujita et al., 2011).  
314 Group IV (DEX+ IV RS): was given DEX plus receiving IV RS solution (1mg/kg). Group V  
315 (DEX+ IN F2): was given DEX plus receiving IN F2 in situ thermoresponsive gel (volume  
316 equivalent to RS 1mg/kg). Group VI (DEX+ IN F4): was given DEX plus receiving IN F4 in situ  
317 thermoresponsive gel (volume equivalent to RS 1mg/kg).

318

### 319 **2.10.3 Biochemical analysis of bone turnover markers**

320 At the end of the study (28 days), animals were killed under general anaesthesia with diethyl ether  
321 after blood collection from retro orbital plexus. Blood was centrifuged at 5000 rpm for 10 min and  
322 serum stored immediately at -20°C for analysis. Serum alkaline phosphatase, serum calcium level,  
323 serum inorganic phosphorus level and serum creatinine were estimated. The values of blood  
324 biochemistry were measured in Ain Shams Laboratory Center, Cairo, Egypt using Roche  
325 biochemical reagents via photometric assay (Hitachi 917 autoanalyzer, Roche Diagnostics, USA)

326

#### 327 **2.10.4 Histological study of bone internal structure**

328 Autopsy samples were taken from the femur bone of rats in different groups and fixed in 10%  
329 formal saline for twenty-four hours then decalcification in formic acid. Washing was done with  
330 water then alcohol was used for dehydration. Specimens were cleared in xylene and embedded in  
331 paraffin wax melted at 56°C in an oven for twenty-four hours. Paraffin tissue blocks were prepared  
332 for sectioning at 4 microns thickness by a sledge microtome (Rotary Leica RM2245, USA). The  
333 obtained tissue sections were collected on glass slides, deparaffinised, stained by hematoxylin &  
334 eosin stain for examination via microscopy (Axiostar Plus, Zeiss, USA) (Banchroft 1996).

335

#### 336 **2.11 Evaluation of nasal cytocompatibility**

337 MTT assays were used to evaluate the *in vitro* cytotoxicity of the prepared gels on Calu-3 cells.  
338 Briefly, cells were seeded at a density of  $1 \times 10^4$  cells/well in 96-well plates and incubated at 37 °C  
339 in a humidified atmosphere (95% RH) with 5% v/v CO<sub>2</sub> (Humid CO<sub>2</sub> incubator, Shel lab 2406,  
340 USA). Seven aliquots of the co-polymers solutions (7.81-15.62-31.25-62.5-125-250-500 µg/mL)  
341 were added to the wells, followed by incubation for another 24 h. Both sodium dodecyl sulphate  
342 treated and untreated cells were used as positive and negative controls respectively. After 24 h of  
343 incubation, 20µL of MTT solution (5 mg/mL in phosphate buffered saline pH 7.4 was added to  
344 each well and incubated at 37°C for 4 h. then, MTT solution was removed and DMSO was added  
345 to solubilize the formed formazan. The absorbance was read at 570 nm on a microplate reader  
346 (Tecan Sunrise®, Switzerland). Cell metabolic activity as a proxy for viability (%) was calculated  
347 according equation 3:

348

$$349 \quad \text{Cell metabolic activity (viability, \%)} = (\text{Absorbance test/Absorbance control}) \times 100 \quad (3)$$

350 Where the absorbance control was the absorbance of untreated cells in the control wells.

351

## 352 **2.12 Statistical analysis**

353 The measured data were shown as mean of 3 or 4 determinations  $\pm$  standard deviation (SD). The  
354 mean values were analysed for their statistical significance using either Student's t test or ANOVA  
355 using Graph Pad InStat software program version 3.06 (GraphPad Software, Inc., USA). p-value  $\leq$   
356 0.05 was chosen as the criterion for statistical significance.

357

## 358 **3. Results and discussions**

### 359 **3.1. Preparation of gel components and telomerization kinetics**

360 Thermoresponsive polymers were synthesised by free radical polymerization using PETMP as a  
361 chain transfer agent. PETMP was chosen as the transfer agent in order to produce star-shaped  
362 polymers. The homopolymers of PEGMA-ME 188 and PEGMA-EE246 were prepared as controls  
363 to establish telomerisation kinetics before being copolymerized with the more hydrophilic  
364 monomer PEGMA-ME475 to adjust the gelation temperature to a value  $> 30^{\circ}\text{C}$  (Fig. S2 and Table  
365 1).

366 The PEGMA based polymers were prepared aiming to combine the advantageous properties of  
367 polyethylene glycol (non-toxic, non-immunogenic) and thermosensitivity in one macromolecule.  
368 Table 1 demonstrates the molar masses and characteristics of the homo- and co-polymers.

369

370

371

**Table 1.** Summary of the polymers synthesized in this study

<b>Polymer structure</b>	<b>Mn (kDa)</b>	<b>PDI</b>	<b>Tc (<math>^{\circ}\text{C}</math>)</b>	<b>PEGMA475 (%)</b>	<b>C<sub>T</sub></b>
Poly(PEGMA188-ME)	38.9	1.9	26	0	0.97
Poly(PEGMA188-ME-co-PEGMA475- ME)	36.2	2.1	32	16	---
Poly(PEGMA246-EE)	35	2.3	25	0	0.93
Poly(PEGMA246-EE-co-PEGMA475-ME)	43	2.2	31	10	---

372

373 The control of polymer architecture through the use of polyfunctional chain transfer agent  
374 in free radical polymerization was investigated, both theoretically and experimentally.  
375 Yuan et al studied the telomerization of methyl methacrylate with the tetrafunctional  
376 transfer agent PETTMP and found a  $C_T$  to be 0.64 (Yuan and Di Silvestro, 1995). It is  
377 assumed that the smaller the value of transfer constant ( $C_T \approx 1$ ) is an indication of  
378 telomerization reaction efficiency (Loubat and Boutevin, 2001; Pardal et al., 2009).  
379 There are several methods of determining the transfer constant ( $C_T$ ) of the transfer agent. The most  
380 widely used one is given by (O'Brien and Gornick, 1955). This method requires a plot of  $\ln[T_0]/[T]$   
381 versus  $\ln[M_0]/[M]$  where  $[T_0]$ ,  $[T]$ ,  $[M_0]$  and  $[M]$  are the molar concentrations of telogen and  
382 monomer at time 0 and time t respectively where the value of  $C_T$  can be determined from the slope  
383 of the line obtained.  
384 This method was applied to the telomerization of PEGMA188-ME and PEGMA246-EE in the  
385 presence of PETTMP as a telogen under nitrogen, in 2-butanone at 70 °C, in the presence of AIBN  
386 as initiator. Each reaction was monitored by sampling, and each aliquot was quenched in ice to  
387 stop the reaction. The monomer (M) and telogen (T) conversions were determined, respectively,  
388 with  $^1\text{H}$  NMR and iodine titration as explained in the experimental section.  
389 From the change of each monomer and telogen concentration in the course of their telomerization,  
390 the  $C_T$  values were determined for Poly(PEGMA188-ME) and Poly(PEGMA246-EE) and found  
391 to be 0.97 and 0.93 respectively, indicating efficient telomerization reaction (Fig. S2).

392

### 393 **3.3 Polymer characterization**

394 The chemical structure of copolymers was characterized by FTIR,  $^1\text{H}$ -NMR, and GPC.

395

#### 396 **3.3.1 FTIR**

397 In order to confirm the formation of the polymers, FTIR spectra were performed as shown in Fig.  
398 S3. In the generated spectra, the bands of 1726 and 1113  $\text{cm}^{-1}$  were respectively assigned to the  
399 C=O and C-O stretching vibrations for carbonyl group of carboxylic ester, the wide band around  
400 3423  $\text{cm}^{-1}$  to the O-H stretching vibration, the bands of 2871  $\text{cm}^{-1}$  and 1470 to 1380  $\text{cm}^{-1}$  to the  
401 stretching and bending vibrations of methylene (-CH<sub>2</sub>). The absence of the acrylate group at the  
402 range of 1620 - 1650  $\text{cm}^{-1}$  indicates that acrylate groups were depleted and hence, the successful  
403 formation of polymers by free radical polymerisation (Lee et al., 2003).

404

### 405 3.3.2 NMR

406 The polymers were characterised by NMR spectroscopy as shown in Fig. S4.

407 <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> using Bruker 400 MHz. The molar fraction of  
408 PEGMA475-ME in Poly(PEGMA246-EE-co-PEGMA475-ME) was calculated from <sup>1</sup>H NMR  
409 spectra by comparing the overall integration of the methoxy protons (3.41 ppm) to the  
410 integration at 1.23 ppm for the 3 terminal protons of ethoxy moiety of PEGMA246-EE.

411

412 Poly(PEGMA188-ME) and Poly(PEGMA188-ME-co-PEGMA475-ME)

413 <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, δ, ppm): 4.25 (-CO-OCH<sub>2</sub>CH<sub>2</sub>), 4.1-4.2 (CCH<sub>2</sub>OCOCH<sub>2</sub>-), 3.5-3.75  
414 (-CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>O-), 3.35-3.41 (CH<sub>3</sub>O-), 2.3-2.5 (OCOCH<sub>2</sub>CH<sub>2</sub>S-), 1.7-2.01 (-SCH<sub>2</sub>C(CH<sub>3</sub>-),  
415 0.72-1.16 (-CH<sub>2</sub>C(CH<sub>3</sub>-).

416

417 Poly(PEGMA246-EE)

418 <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, δ, ppm): 4.22 (-CO-OCH<sub>2</sub>CH<sub>2</sub>), 4.1 (CCH<sub>2</sub>OCOCH<sub>2</sub>-), 3.5-3.7 (-  
419 CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>O-), 3.4 (CH<sub>3</sub>CH<sub>2</sub>O-), 2.2 (OCOCH<sub>2</sub>CH<sub>2</sub>S-), 1.62-2.06 (-SCH<sub>2</sub>C (CH<sub>3</sub>-), 1.23  
420 (CH<sub>3</sub>CH<sub>2</sub>O-), 0.72-1.16 (-CH<sub>2</sub>C (CH<sub>3</sub>-).

421

422 Poly(PEGMA246-EE-co-PEGMA475-ME)

423 <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, δ, ppm): 4.3 (-CO-OCH<sub>2</sub>CH<sub>2</sub>), 4.1 (CCH<sub>2</sub>OCOCH<sub>2</sub>-), 3.5-3.7 (-  
424 CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>O-), 3.47 (CH<sub>3</sub>CH<sub>2</sub>O-), 3.41 (CH<sub>3</sub>O-), 2.3-2.45 (OCOCH<sub>2</sub>CH<sub>2</sub>S-), 1.62-2.06 (-  
425 SCH<sub>2</sub>C (CH<sub>3</sub>-), 1.23 (CH<sub>3</sub>CH<sub>2</sub>O-), 0.72-1.16 (-CH<sub>2</sub>C(CH<sub>3</sub>-).

426

### 427 3.3.3 Gel permeation chromatography (GPC)

428 Fig. S5 and Table 1 present the molar masses relative to polystyrene standards for the respective  
429 polymers. The polymers synthesised had a large polydispersity index, as expected for polymers  
430 prepared by free radical polymerization methods. Conventional free radical methods were used  
431 since they do not require complex conditions or metals, employed in controlled polymerisations  
432 that might be difficult to eliminate and which could otherwise present a source of toxicity (Johnson  
433 et al., 2008) when used with cells (Chen et al., 2006). Prior reports have shown that high  
434 polydispersity polymers can still form thermogelling solutions (Lietor-Santos et al., 2009).

435

436

### 437 **3.6. Rheological property**

438 Gels made from the prepared polymers were characterised by experiments to measure their  
439 elastic/storage ( $G'$ ) and the viscous ( $G''$ ) moduli. The variation of  $G'$  with temperature was  
440 apparent in temperature/gel moduli plots of a representative gel (Fig. S6A). This could confirm  
441 the high dependency of intermolecular attraction between polymer chains, and hence the gel  
442 elasticity, on temperature. Below the Lower Critical Solution Temperature (LCST) of the polymers  
443 (as estimated from the cloud point), they behaved as viscous fluids with  $G'' > G'$ . At temperature  
444 values around the LCST, a crossover occurred, with  $G' > G''$ , indicative of gel formation. Both  
445 moduli increased with temperature, with  $G'$  remaining higher than  $G''$ .

446 The ability to manipulate the properties of the gel by changing the thermoresponsive polymer is  
447 shown in Fig. S6B. For equivalent composition ratios, gels formed above LCST using  
448 homopolymers were of higher moduli than copolymers containing PEGMA475. The rheology  
449 experiments thus indicated that the mechanical properties of the gels were able to be tuned by  
450 composition, suggesting a simple way of tailoring the gel properties for a particular application.

451

### 452 **3.3.4. Cloud point determination**

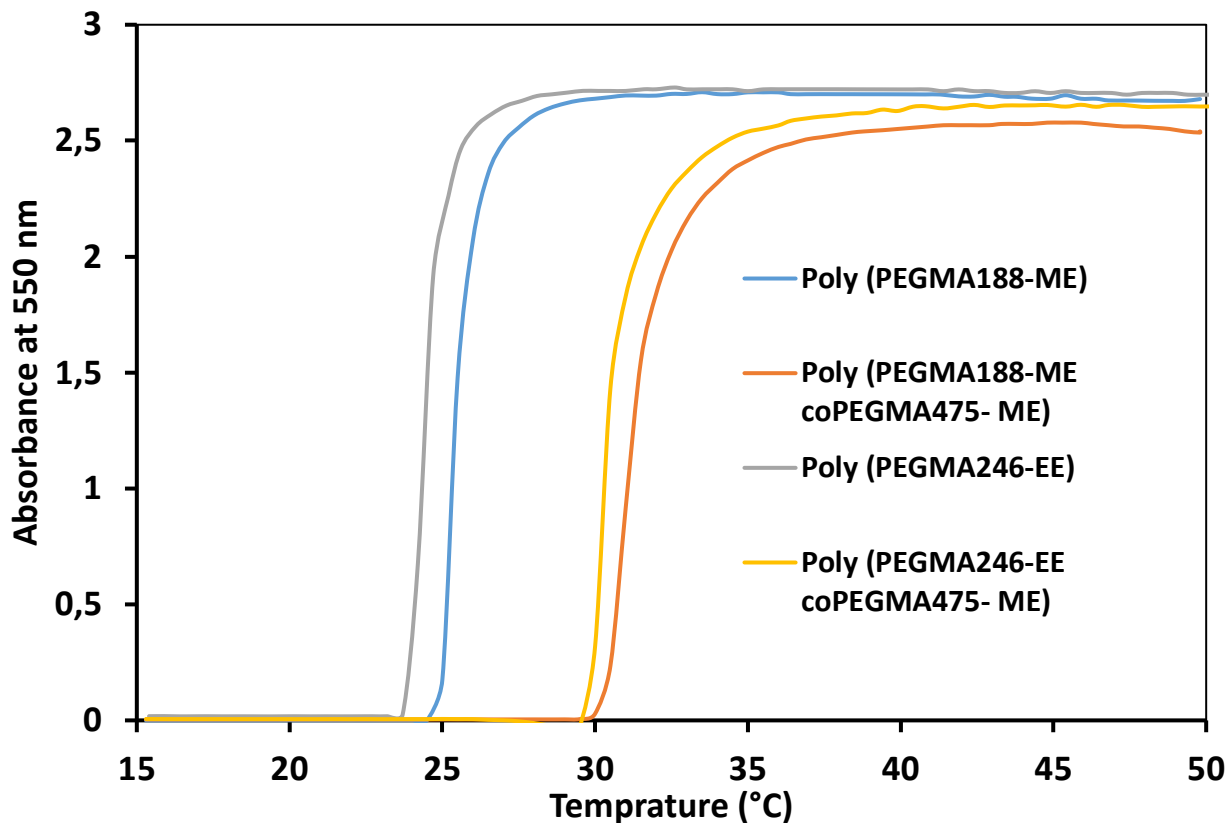
453 Thermoresponsive polymers undergo a coil-to-globule transition at the lower critical solution  
454 temperature (LCST) and become water insoluble (Liu et al., 2009). When the polymers are in the  
455 collapsed state their solution becomes turbid as a result of polymer chain aggregation.

456 The solution turbidity starts at the cloud point temperature ( $C_{pt}$ ) and this can be easily determined  
457 experimentally by light transmission measurement. Cloud point temperatures were measured to  
458 estimate the LCST for each polymer (Alava and Saunders, 2006) as shown in table 1.

459 Phase transitions temperatures of the polymers were modulated by copolymerization of  
460 hydrophobic OEGMA component with the hydrophilic PEGMA monomer (Table 1, Fig. 1). We  
461 were interested in polymers that would exhibit phase transitions at, or close to, body temperature.  
462 It was found that the statistical FRP copolymer of OEGGMA-co-PEGMA (95/5 monomer-feed  
463 ratio) met this criterion. As expected, the cloud point temperatures were higher for PEGMA-  
464 ME475 copolymers than homopolymers, owing to the presence of hydrophilic monomer.  
465 PEGMA-ME475 had the effect of facilitating polymer chain dehydration to happen at higher cloud



466 point value. Similar cloud point values have been reported previously for a number of linear  
467 thermosensitive polymers (Magnusson et al., 2008). Hence, Poly(PEGMA188-ME-co-  
468 PEGMA475-ME) and Poly(PEGMA246-EE-co-PEGMA475-ME) were suitable for drug loading  
469 and its nasal delivery.



470  
471

472 **Fig. 1.** Cloud point measurements of polymers using UV–visible turbidimetry experiments at 550 nm in  
473 water (1 mg/mL) versus temperature.

474

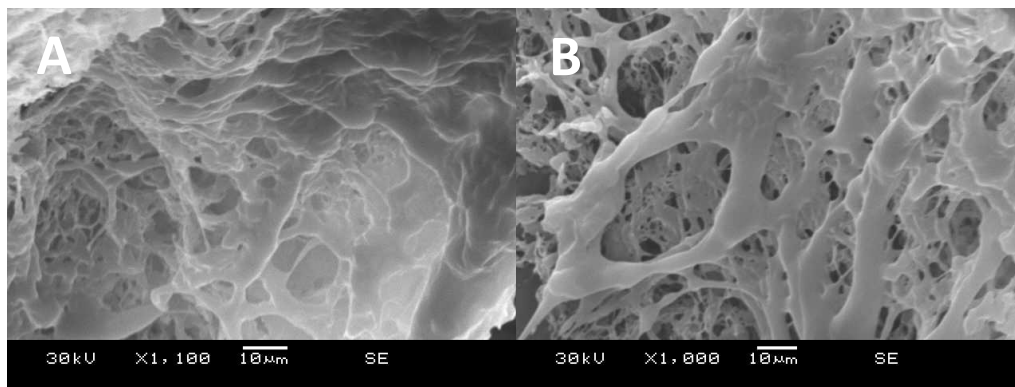
### 475 3.4 SEM imaging

476 Fig. 2 depicts typical SEM micrographs of freeze-dried Poly(PEGMA188-ME-co-PEGMA475-  
477 ME) and Poly(PEGMA246-EE-co-PEGMA475-ME) hydrogels the microstructure of the interior  
478 of the freeze-dried hydrogels was revealed. Poly(PEGMA188-ME-co-PEGMA475-ME) hydrogels  
479 had relatively regular pores of tens to hundreds of micrometres in size formed through well-  
480 connected gel matrices.

481 The morphology of Poly(PEGMA246-EE-co-PEGMA475-ME) appeared somewhat different,  
482 where an irregular, entangled fibrous morphology is noticed. It is obvious that gel matrices

483 appeared porous with channels/voids of 5–20  $\mu\text{m}$ . Calculations indicated that this void volume  
484 was equivalent to 60–75% porosity in the gels studied.

485 The porous network structure of these hydrogels with their biodegradability and relatively strong  
486 gel strength suggested that these materials might be useful as drug delivery biomaterials the high  
487 internal surface areas with low diffusional resistance would be favorable for small drug molecules  
488 like RS to move freely in the polymeric network.



489

490 **Fig. 2.** Representative Scanning Electron Micrograph of (a) F2 and (b) F4.

491

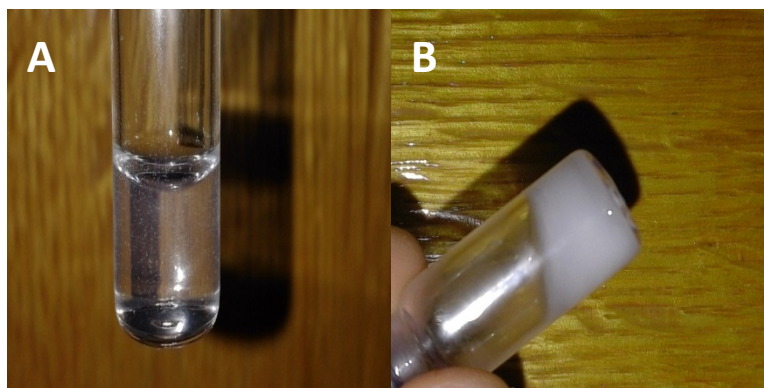
### 492 **3.5. Incorporation of RS in thermoresponsive gels and pH determination**

493 Based on a preliminary study, the co-polymer solutions (Poly(PEGMA188-ME-coPEGMA475-  
494 ME) and Poly(PEGMA246-EE-coPEGMA475-ME)) at 30% were chosen, owing to their  
495 capability to form hydrogels upon heating in a water bath at 37°C. The developed in situ gels were  
496 loaded with RS (0.2% w/v) by simple mixing (F2 and F4 respectively). The image of pre-gelled  
497 solution and formed hydrogel are shown in Fig. 3.

498 The pH of nasal formulations should be such that the formulation will be stable at that pH and at  
499 the same time there would be no irritation to the patient upon administration. Tolerable non-  
500 irritable nasal formulations should have pH range in between 4.5 to 6.5. The pH values of  
501 hydrogels were found to be within the physiologic range of nasal application amounting of  $5.55 \pm$   
502  $0.02$  and  $5.79 \pm 0.04$  for F2 and F4, respectively.

503

504



505  
506 **Fig. 3.** Sol-gel phase transitions of F2 in (a) a sol state at 25 °C, (b) gel state at 37 °C.

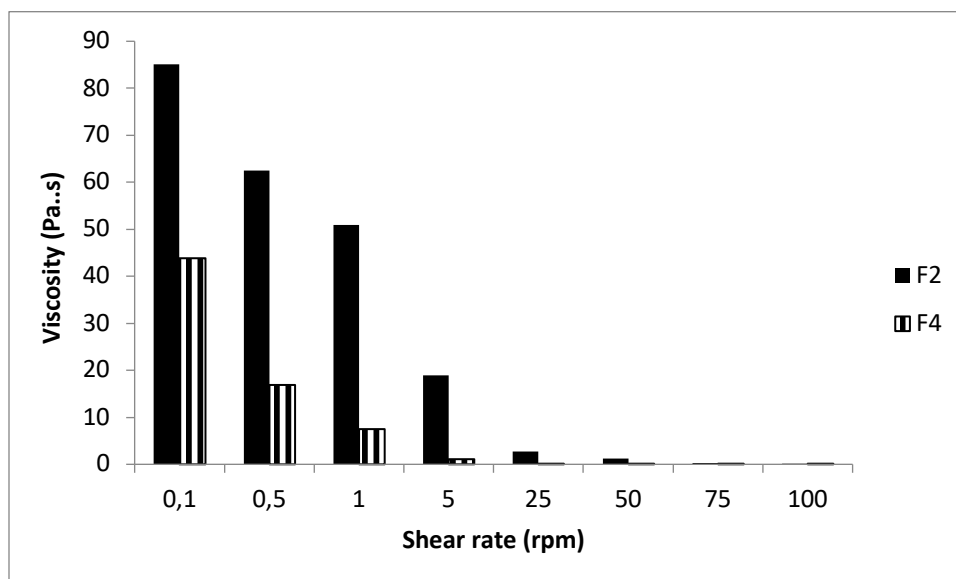
507

508

### 509 **3.6 Viscosity determination of RS loaded thermoresponsive gels**

510 Concerning rheological measurements, the rheograms obtained for the two investigated gels at  
511 37°C displayed the pseudoplastic and shear-thinning behaviour (Fig. 4). Such rheological patterns  
512 could therefore be preferred so that the viscosity decreases, facilitating the flow of the in-situ gel  
513 upon application for intranasal use.

514



515

516 **Fig. 4.** Viscosity measurements of the investigated gel formulations. Data are reported as the mean of three  
517 independent experiments.

518

### 519 **3.7 Bioadhesion strength of RS loaded thermoresponsive gels**

520 When considering nasal delivery, it is highly desirable to formulate bioadhesive delivery vehicles  
521 to enhance their localization and retention time in the nasal area, thus intensifying the contact with  
522 nasal mucosa for the drug absorption. This was the basis for investigating the mucoadhesive  
523 properties for the prepared formulae in this study. The viscosity of the prepared formulae plays an  
524 important role in determining the mucoadhesive properties (Suvannasara et al., 2013). Therefore,  
525 in this work, mucoadhesive force between the interacting polymers (F2 and F4) and the mucin  
526 were elucidated based on viscosity data.

527 The results indicated rheological synergism and promising mucin-thermoresponsive gels  
528 association. The viscosity values of mucin-gels mixtures showed values of  $1.85 \pm 0.07$  and  $1.64 \pm$   
529  $0.016$  Pa·s for F2 and F4 respectively. Such values were revealed to be higher than the sum of the  
530 corresponding viscosity values of the separate components of mucin ( $1.14 \pm 0.11$  Pa·s) and  
531 individual co-polymers (respective  $0.10 \pm 0.04$  and  $0.096 \pm 0.008$  Pa·s values for  
532 Poly(PEGMA188-ME-co-PEGMA475-ME) and Poly(PEGMA246-EE-co-PEGMA475-ME).  
533 Furthermore, F2 exhibited a significantly higher component of mucoadhesion ( $0.6$  Pa·s), almost  
534 1.51fold higher than that for F4 ( $0.39$  Pa·s) ( $P < 0.001$ ). Therefore, such formulations were  
535 considered potential mucoadhesive intranasal drug delivery systems.

536 It was found that incorporating high molecular weight PEG based polymers onto the surfaces of  
537 hydrogels and hydrogel microparticles enhanced mucoadhesion. PEG chains are able to  
538 interpenetrate and associate with mucin by forming hydrogen bonds with its carbohydrate regions  
539 hence acting as a 'glue' (Wang et al., 2008) (Maisel et al., 2016).

540

### 541 **3.8 In vitro drug release**

542 RS was utilized as a hydrophilic model drug to evaluate the feasibility of F2 and F4 for its sustained  
543 delivery. RS has a log octanol/water partition-coefficient of  $-3.48$  (Biernacka et al., 2013). Free  
544 RS reached 100% release within 4 hours owing to its high polarity and hydrophilicity, similar to  
545 that previously reported (Nasr et al., 2011). The release profile of RS from hydrogels is  
546 investigated as shown in Fig. 5. The data indicates that 63 and 61.5 % of RS was released in 2 days  
547 for F2 and F4 respectively under sink conditions. Such similar percent RS release was confirmed  
548 by similarity factor calculated value ( $f_2 = 72.37$ ).

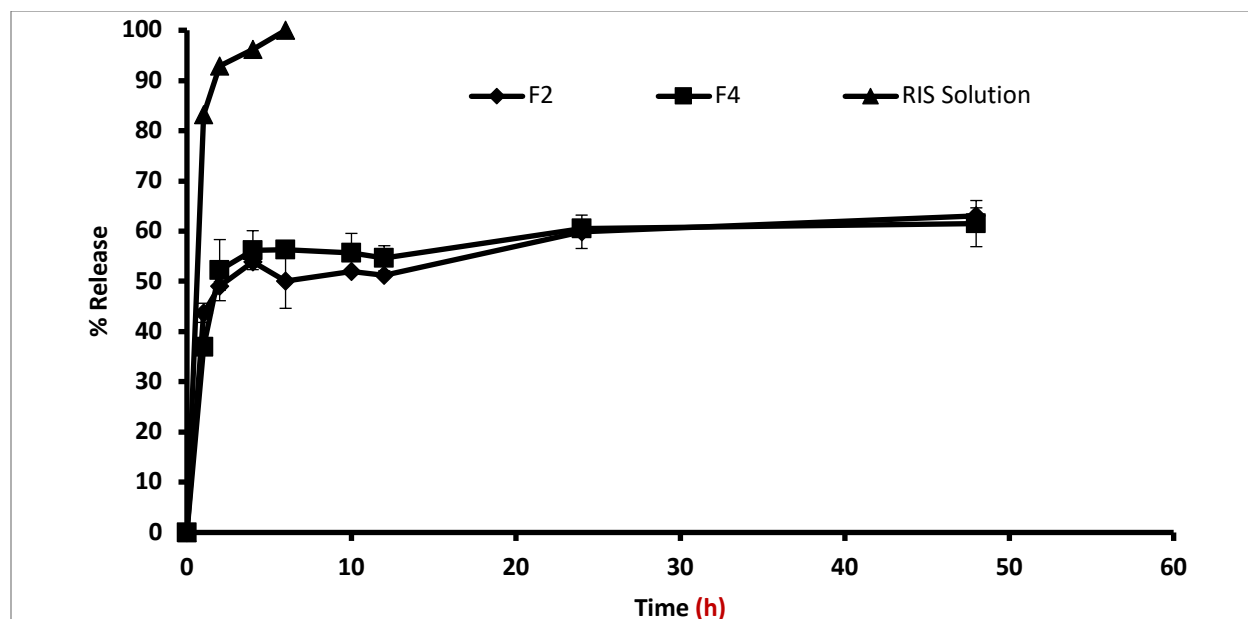
549 RS exhibited an initial burst release from both hydrogel matrices reaching values of 43.7 and 37  
550 % at first hour respectively followed by slowed and sustained release rate till the end of  
551 experiment. The burst release of RS release was more likely due to the presences of RS on the  
552 surface or distributed in the hydrogels network during the gelation process. Besides, the  
553 hydrophilic RS might possess a higher tendency to partition in the hydrophilic domain of the  
554 hydrogel and to diffuse rapidly and easily through the hydrogel into the release medium. The  
555 porous network of the prepared matrices, observed in SEM images, confirmed the previous  
556 hypothesis, indicating the diffusion-controlled drug release.

557 After that, the entrapped RS into the hydrogel was released slowly due to the swelling and  
558 degradation of the hydrogel. Because the hydrogel did not degrade completely during the time of  
559 investigation, some part of the RS entrapped in the inner core of the hydrogel would not be released  
560 until the hydrogel was degraded entirely.

561 It is worthy to note that the prepared hydrogels (F2 and F4) exhibited a wide range of erosion rates  
562 and maintained their mass for over two days. The percentage of gels that remained after 2 days  
563 was 25 and 30% for F2 and F4, respectively (data not shown). F4 showed significantly higher  
564 resistance against mass erosion in aqueous solution as compared to F2. This might be attributed to  
565 its content of the longer chain length of PEGMA246-EE. Higher viscosity of the in situ platform  
566 might also slow the degradation. Consequently, such in situ gels seemed to be more functional for  
567 a sustained RS delivery.

568 A similar release profile was observed for an intranasal thermosensitive hydrogel utilizing  
569 quaternized chitosan and poly(ethylene glycol) (Wu et al., 2007). Such a biphasic release pattern  
570 was a common finding seen in previous research papers studying the release of different  
571 biopharmaceuticals from thermosensitive hydrogels (Rangabhatla et al., 2016; Wu et al., 2007).

572



573

574 **Fig. 5.** In vitro release profiles of RS and RS loaded gel formulations over 48 h. Data are reported as the  
 575 mean  $\pm$  S.D. of three independent experiments.

576

### 577 3.9. Ex-vivo permeation

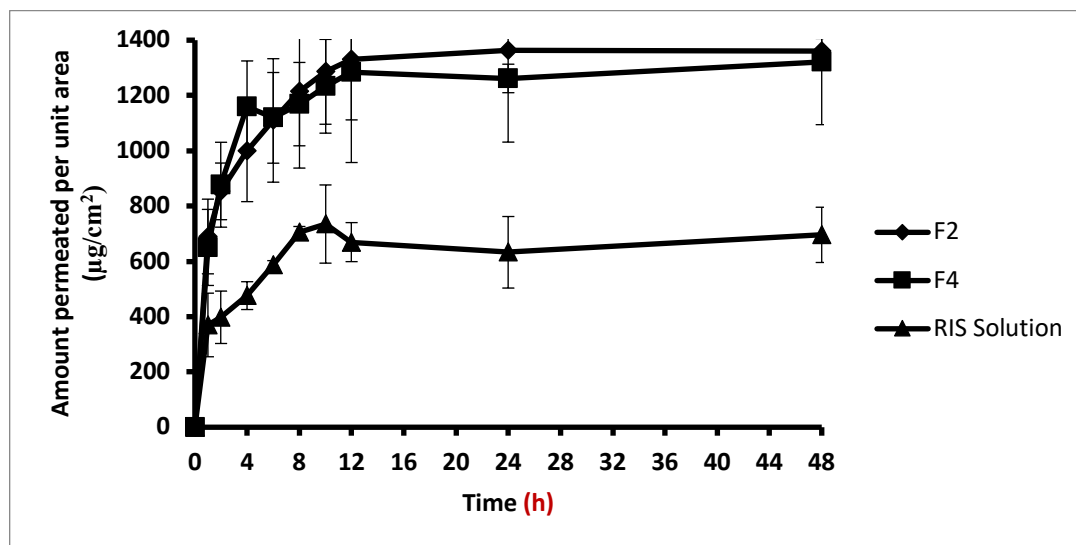
578 Fig. 6 illustrates the amount of RS permeated per unit surface area of the sheep nasal mucosa  
 579 versus time for F2 and F4, using RS solution as a comparison. RS-loaded formulae exhibited  
 580 higher permeation as compared to RS solution. According to the biopharmaceutics classification  
 581 system, RS belongs to class III (high solubility/low permeability) (Fazil et al., 2016; Jung and Han,  
 582 2014).

583 Permeation enhancement potential of the two investigated formulations could be due to the  
 584 surfactant properties of PEG ethers in the polymers (Casiraghi et al., 2015), possibly solving the  
 585 permeability problem and low flux of RS (Nam et al., 2011). Additionally, the hydrogels took  
 586 more time for degradation in the biological system which showed permeation over 48 h. Moreover,  
 587 the mucoadhesive capability of the thermogels might contribute to nasal retention which plays a  
 588 very important role in transnasal penetration promoting effects.

589 The flux for F2 and F4 was found to be 70.9 and 94  $\mu\text{g}/\text{cm}^2\text{h}^{-1}$ , respectively, while only flux value  
 590 of 43.52  $\mu\text{g}/\text{cm}^2\text{h}^{-1}$  was depicted for RS solution, proving that the RS containing formulae  
 591 possessed high penetration through the nasal mucosa. Similar to what was obtained in the in vitro

592 release experiment, there was no statistically significant difference between the cumulative amount  
 593 of RS from F2 and F4 permeated after 48 h ( $p > 0.05$ ) (Maisel et al., 2016).

594



595

596 **Fig. 6.** Ex vivo permeation of RS from its solution and RS loaded gel formulations. Data are reported as  
 597 the mean  $\pm$  S.D. of three independent experiments.

598

599

### 600 3.10 In vivo study

601 **Table 2.** Biochemical analysis of serum levels of bone turnover markers for the different treatment groups. Data are  
 602 reported as the mean  $\pm$  S.D. of four independent experiments.

Experimental Group	Treatment	ALP (IU/l)	Serum Calcium (Ca) (mg/dl)	Serum Phosphorus (P) (mg/dl)	Serum creatinine (mg/dl)
Group I	IN saline	144.5 $\pm$ 23.3	9.7 $\pm$ 0.07	8.2 $\pm$ 0.3	0.3 $\pm$ 0.05
Group II	SC DEX	383 $\pm$ 111.7	8.6 $\pm$ 0.2	3.7 $\pm$ 3.3	0.5 $\pm$ 0.07
Group III	DEX+IN RS	304.5 $\pm$ 3.5	9.8 $\pm$ 0.4	8.9 $\pm$ 1.6	0.3 $\pm$ 0.05
Group IV	DEX+IV RS	217 $\pm$ 125.9	9.9 $\pm$ 0.3	9.4 $\pm$ 2.4	0.3 $\pm$ 0.26
Group V	DEX+IN F2	206 $\pm$ 62.2	10.1 $\pm$ 0.4	9 $\pm$ 1.4	0.3 $\pm$ 0.05
Group VI	DEX+IN F4	271 $\pm$ 145.7	9.8 $\pm$ 0.1	9.2 $\pm$ 1.6	0.4 $\pm$ 0.05

603

604 In this study, an osteoporosis model was induced by SC administration of toxic doses of  
605 dexamethasone sodium phosphate (DEX) in experimental rats. DEX, as a glucocorticoid, has the  
606 potential to compromise the skeletal integrity, decrease osteoblastic activity and promote  
607 osteoblast and osteocyte apoptosis, resulting in increased fractures risk and augmented bone loss  
608 (O'Brien et al., 2004; Yao et al., 2008).

609 Following induction of osteoporosis and RS treatment, biochemical markers, namely, alkaline  
610 phosphatase (ALP), calcium, inorganic phosphorus and creatinine, were tested. Serum ALP is an  
611 osteoblast-related protein that directly promotes osteoid formation and mineralization. It is a bone  
612 formation marker that used to indicate metabolic bone disease (Garnero and Delmas, 1993;  
613 Regidor et al., 2008). Serum calcium and phosphorus are also markers of osteoporotic bone and  
614 bone mineral content and creatinine is a marker of Bone resorption (Fazil et al., 2016).

615 Table 2 depicts the biochemical examination of serum levels of bone turnover markers. Group-I,  
616 which acted as a negative control, exhibited normal serum levels of ALP and creatinine amounting  
617 to  $144.5 \pm 23.3$  (IU/l) and  $0.3 \pm 0.05$  (md/dl) respectively. On the other hand, the toxic group II,  
618 receiving SC DEX alone, showed a marked elevation in the serum levels of ALP and creatinine  
619 with respective values of  $383 \pm 111.7$  (IU/l) and  $0.5 \pm 0.07$  (mg/dl), the highest among all the  
620 tested groups, confirming the induction of GIO.

621 This was reversed by RS in all RS treated groups (groups III-VI). A significant reduction in ALP  
622 and creatinine serum levels below the toxic level were demonstrated confirming promising  
623 recovery and capability of decreasing GIO risk. A notable significant increase of these values was  
624 obtained as compared to those of normal group (group I) ( $p < 0.05$ ).

625 Concerning serum calcium and phosphorus levels, the toxic group exhibited significantly reduced  
626 values among all the tested groups, proving osteoporosis formation. Following induction of  
627 osteoporosis, fragile conditions of the bone led to the release of calcium and phosphorus and their  
628 urinary excretion and hence, their reduced absorption (Fazil et al., 2016). All treated groups with  
629 different forms of RIS showed higher calcium and phosphorus levels, proving the ability of RS to  
630 enhance the absorption of calcium and its bone deposition and to prevent bone-breaking tendency.

631

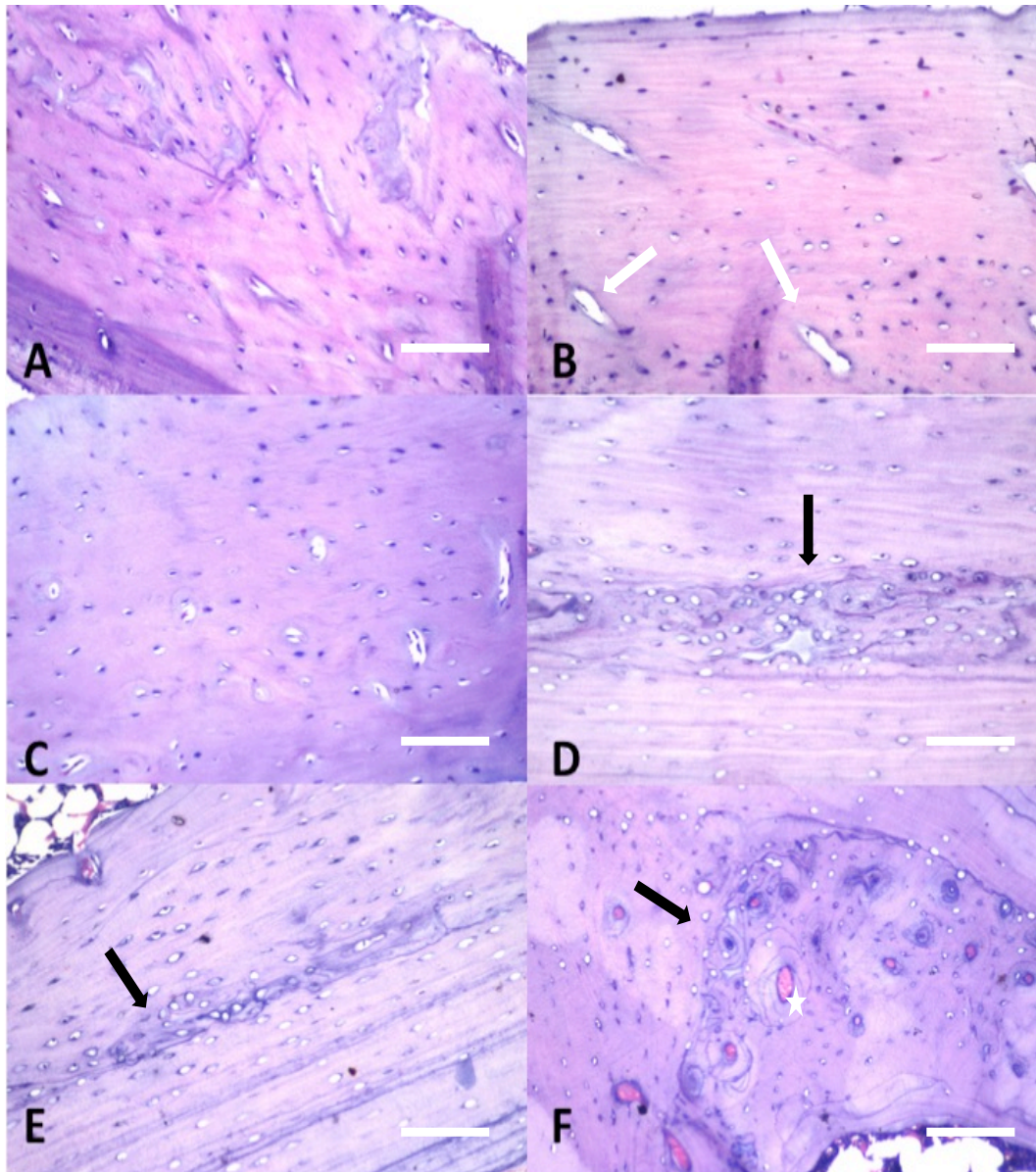
### 632 **3.11 Histopathological examination**

633 Histopathological examination of bones of animals of group I (normal control) (Fig. 7a) showed  
634 no histopathological alteration and the normal histological structure of the osteoblasts. The toxic



635 group II histological manifestations exhibited focal areas of resorption with appearance of cavities  
636 and multiple osteoclasts as indicated by white arrows (Fig.7b), confirming osteoporosis induction.  
637 There were focal areas of resorption with few osteoclasts noticed in histological features in rats of  
638 group III treated with IN solution. The histopathology of bone was quite similar to the picture in  
639 rats of group IV, treated with IV solution, exhibiting focal area of osteogenesis with remodeling  
640 of the osteoblasts (Fig.7 c and d).  
641 As for rats of group V treated with F2, there were focal area of osteogenesis with osteoblasts  
642 remodeling as well as formation of dark basophilic lines of bone deposition as indicated by black  
643 arrows. The histopathological picture mirrored the results of treatment of osteoporosis (Fig.7e).  
644 Group VI treated with F4 shows the ultimate treatment as manifested by the angiogenesis of newly  
645 blood capillaries with dark basophilic line of bone deposition as indicated by white star. In  
646 addition, osteoblasts arrangement was recorded (Fig.7f), achieving promising recovery following  
647 osteoporosis induction.  
648 The prepared in situ gels were superior in their inhibitory effect as compared to IN and IV  
649 solutions, possibly due to proven low permeability of the hydrophilic drug. In addition, as for IN  
650 solution, the expected rapid wash out owing to nasal mucociliary clearance might contribute to its  
651 lower antiosteoporotic capability (Fazil et al., 2016).

652



653  
 654 **Fig.7.** Light micrograph of rat internal structure of bone treated with (a) IN saline, (b) SC DEX, (c) DEX+IN RS, (d)  
 655 DEX+IV RS, (e) DEX+F2 and (f) DEX+F4. White arrows indicate focal areas of resorption with appearance of  
 656 cavities, black arrows indicate focal area of osteogenesis and white star indicates angiogenesis of newly blood  
 657 capillaries (White bar 100  $\mu$ m).

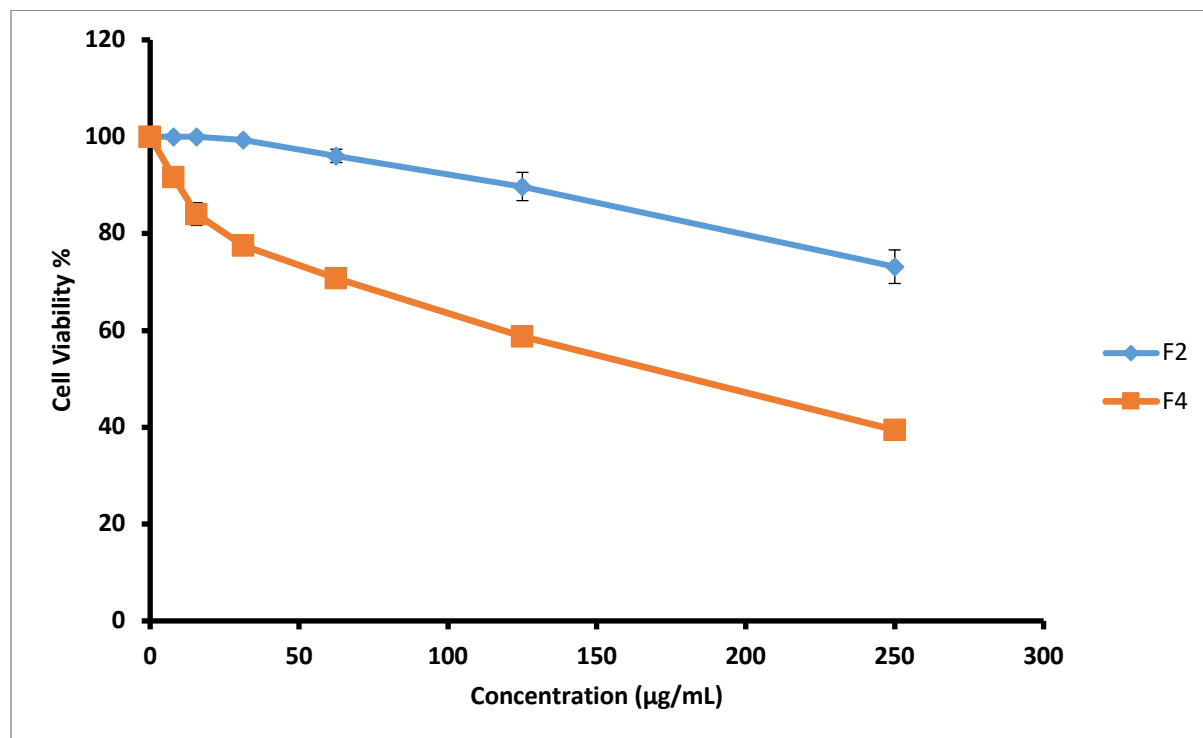
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### 660 3.12 Evaluation of nasal cytocompatibility

661 Mucoadhesive thermoresponsive hydrogels must be capable of delivering drugs in a sustained  
662 manner without compromising the host cell viability following nasal administration. In this regard,  
663 the effect of the prepared hydrogels on the cell viability of Calu-3 cells was investigated. Good  
664 cytocompatibility was observed for F2 and F4 with no apparent cytotoxicity against the tested  
665 cells (cell viability >75%) after 24-h incubation at concentrations up to 250 and 62.5  $\mu\text{g}/\text{mL}$   
666 respectively (Fig. 8). It was only at hydrogel concentrations of 125 and 250  $\mu\text{g}/\text{mL}$  that the  
667 cytotoxicity of F4 was significantly reduced. Lower cytocompatibility values seen with F4 could  
668 be associated with its content of longer chains of PEGMA246-EE, possibly greatly interfering with  
669 the cell viability. Such values were comparable with other PEGMA-bearing copolymers utilized  
670 recently for drug delivery (Blanco-Fernandez et al., 2017). In addition, nonlinear PEG analogues  
671 had not induced cell death, even at a concentration as high as 10 mg /mL (Lutz, 2008).

672



673

674 **Fig. 8.** Viability of Calu-3 cells by MTT assay after incubation with various concentrations of the test  
675 samples at 37 °C. Data are reported as the mean  $\pm$  S.D. of three independent experiments.

676

677

#### 678 **4. Conclusion**

679 We suggest that the developed thermoresponsive in situ gels can contribute to the successful nasal  
680 delivery of RS. The characterization of the prepared in situ gels and the results of release, ex-vivo  
681 permeation experiments showed favourable rheological properties, mucoadhesive potential,  
682 sustained drug release and good permeability enhancement. Non-irritating pH value and  
683 cytocompatibility of in situ gels confirmed their reasonable nasal tolerability. Moreover, better in  
684 vivo inhibitory effect of RS-loaded in situ gels was achieved compared to IN and IV RS solutions  
685 on osteoporosis induction in experimental rats. Hence, these newly developed in situ gel  
686 formulations have characteristics which are appropriate for mucoadhesive thermoresponsive  
687 delivery systems.

688

#### 689 **5. Acknowledgment**

690 This research was supported by the Egyptian Government Scholarship to MES. We also thank Dr  
691 Aram Saeed for experimental support and helpful discussions.

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