OSTEOGENIC EFFICIENCY OF *IN SITU* GELLING POLOXAMINE SYSTEMS WITH AND WITHOUT BONE MORPHOGENETIC PROTEIN-2[§]

Ana Rey-Rico[1], Maite Silva[2], José Couceiro[2], Angel Concheiro[1] and Carmen Alvarez-Lorenzo[1,*]

[1] Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia,
[2] Instituto de Ortopedia y Banco de Tejidos Musculoesqueléticos, Universidad de Santiago de Compostela,
15782-Santiago de Compostela, Spain

Abstract

In situ gelling solutions for minimally invasive local application of bone growth factors are attracting increasing attention as efficient and patient-friendly alternative to bone grafts and solid scaffolds for repairing bone defects. Poloxamines, i.e., X-shaped poly(ethylene oxide)-poly(propylene oxide) block copolymers with an ethylenediamine core (Tetronic®), were evaluated both as an active osteogenic component and as a vehicle for rhBMP-2 injectable implants. After cytotoxicity screening of various poloxamine varieties, Tetronic 908, 1107, 1301 and 1307 solutions were chosen as the most cytocompatible and their sol-to-gel transitions were rheologically characterized. Viscoelastic gels, formed at 37 ºC, sustained protein release under physiological-like conditions. Formulations of rhBMP-2 led to differentiation of mesenchymal stem cells to osteoblasts, quantified as alkaline phosphatase activity with a maximum at day 7, and to mineralized nodules. Interestingly, poloxamine solely gels led to an initial proliferation of the mesenchymal stem cells (first week), followed by differentiation to osteoblasts (second to third week). Histochemical analysis revealed that Tetronic 908 is only osteoinductive; Tetronic 1107 is mostly osteoinductive, although its use leads to a minor differentiation to adipocytes; Tetronic 1307, solely or loaded with rhBMP-2, causes differentiation of both osteoblasts and adipocytes. Enhanced expression levels of CBFA-1 and collagen type I were observed for Tetronic 908, 1107 and 1307, both solely and combined with rhBMP-2. The intrinsic osteogenic activity of poloxamines (not observed for Pluronic F127) offers novel perspectives for bone regeneration using minimally invasive procedures (i.e., injectable scaffolds) and overcoming the safety and the cost/ effectiveness concerns associated with large scale clinical use of recombinant growth factors.

Keywords: BMP-2, poloxamine, osteogenic induction, bone regeneration, scaffold, controlled release, injectable hydrogel, PEO-PPO block copolymers, *in situ* gelling.

*Address for correspondence:
Carmen Alvarez-Lorenzo
Departamento de Farmacia y Tecnología Farmacéutica,
Facultad de Farmacia,
Universidad de Santiago de Compostela,
15782-Santiago de Compostela, Spain

Telephone Number: 34 981563100 (14876)
FAX Number: 34 981547148
E-mail: carmen.alvarez.lorenzo@usc.es

Introduction

Bone and cartilage regeneration has become a hot research topic in the past decade (Puppi *et al.*, 2010). The high prevalence of degenerative diseases as well as accidental fractures prompted the search of alternatives to the autogenous and allogeneic grafts. Tissue engineering approaches aim to develop scaffolds that can induce and guide cell proliferation, differentiation and new tissue formation at the site of tissue injury or defect. The scaffolds contain cells or biomolecules that can attract cells and growth factors – small molecules or peptides that promote cell survival and tissue regeneration (Kim and Valentini, 2002; Schoichet, 2010). Bone morphogenetic proteins (BMPs) from bone matrix regulate the differentiation and function of the cells involved in bone and cartilage formation, including osteoblasts and chondrocytes (Urist, 1965; Cowan *et al.*, 2005; Reddi, 2005). Most clinical trials have focused on the use of human recombinant BMP-2 (rhBMP-2) since it is one of the most potent inductors of bone formation *in vivo* (Cheng *et al.*, 2003; Takahashi *et al.*, 2005; Bishop and Einhorn, 2007; Bessa *et al.*, 2008a). In several types of cell cultures, BMP-2 increases the expression of alkaline phosphatase (ALP), osteopontin, osteocalcin, and collagen type I (Jeon *et al.*, 2007). However, BMP-2 administered in solution is quite unstable, rapidly loses its bioactivity and is not retained at the application site long enough for the effective healing of fractures in large animals (Seeherman *et al.*, 2004). The elimination half-life of BMP-2 in nonhuman primates has been found to be about 6-7 min (Poynton and Lane, 2002). Therefore, the design of delivery systems able to protect growth factors from premature degradation, to retain them at the bone injury or defect, and to sustain their release is a clinical requirement (Hsieh *et al.*, 2006; Jeon *et al.*, 2007; Bishop and Einhorn, 2007). An ideal BMP delivery system should be biocompatible, biodegradable or bioeliminable, malleable, easy to handle and sterilizable and should act as a structural template to fill the tissue lesion (Geiger *et al.*, 2003; Seeherman and Wozney, 2005; Issa *et al.*, 2008).

Most scaffolds are solid matrices based on collagen type I, since it is the most abundant component of the extracellular bone matrix synthesized by osteoblasts (Winn *et al.*, 1999). Collagen sponges loaded with rhBMP-2 and/or rhBMP-7 were given FDA approval in 2002 for use in non-union, open tibial fractures and spinal fusions (McKay *et al.*, 2007; White *et al.*, 2007). The main limitations of collagen matrices are their high degradation rate and their poor capability to prevent free diffusion of the growth factors, resulting in a high
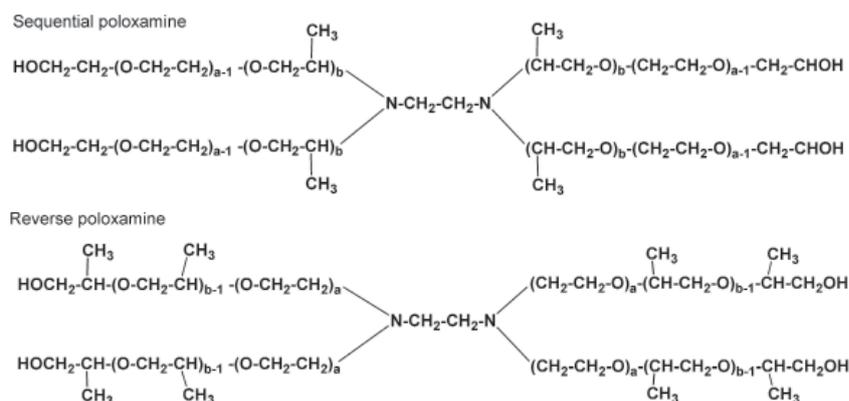
Fig. 1. Structure of sequential and reverse-sequential poloxamines.

initial burst release (Helm *et al.*, 1997; Takahashi *et al.*, 2005). Alternative materials such as gelatin/β-tricalcium phosphate (Yang *et al.*, 2005), poly(L-lactic acid) (PLA) (Kim *et al.*, 2008), poly(L-lactic-co-glycolic acid) (PLGA) (Jeon *et al.*, 2007; Shen *et al.*, 2009), hyaluronic acid (Kim and Valentini, 2002), fibrin gel (Itosaka *et al.*, 2009) or porous hydroxyapatite composites (Sopyana *et al.*, 2007) are under study. The recent developments in the research of BMP delivery carriers have been comprehensively revised by Bessa *et al.* (2008a,b). The use of organic solvents or the high temperature conditions required for preparing hydrophobic matrices (Bessa *et al.*, 2008b) and the deleterious effect of the degradation substances from certain polymers, such as polylactic acid (Puppi *et al.*, 2010), may compromise the stability of the BMPs and the healing of the bone. As an alternative to the preformed implantable scaffolds, injectable bone-filling materials that enable minimally invasive management of the bone defects are gaining increasing attention (Puppi *et al.*, 2010).

Temperature-sensitive block copolymers bearing poly(ethylene oxide), PEO, are endowed with features that mimic those of the extracellular matrix whilst enabling the formulation of aqueous systems that are easily syringeable at room temperature and lead to highly viscoelastic gels at 37 ºC (Lutolf *et al.*, 2003a,b; Pratt *et al.*, 2004; He *et al.*, 2008; Yu and Ding, 2008). The low surface tension of the gels enables the loading of proteins in the aqueous phase of the hydrogel without denaturing, while the sol-to-gel transition makes the sustained release under physiological conditions possible (Bromberg and Ron, 1998; Kabanov and Alakhov, 2002). Although still few, the studies carried out with polypropylene fumarate-PEG (Fisher *et al.*, 2004) and poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), PEO-PPO-PEO, poloxamer (Pluronic®) triblock copolymers (Issa *et al.*, 2008) evidenced the ability of *in situ* gelling systems to regulate the delivery of BMP-2 and BMP-7. Comparatively to biodegradable polymers, such as PLA or PLGA, which are not completely reabsorbed and radiographically appear as voids or black holes in the formed bone, poloxamer can be completely replaced by the new bone (Clokie and Urist, 2000). Once in blood, poloxamers are rapidly excreted in the urine, with minor accumulation in lung, liver, brain, and kidney tissue, and no risk of reproductive and/or developmental toxicity has been reported (Cosmetic Ingredient Review (CIR) Expert Panel, 2008).

Small synthesizable organic molecules, such as simvastatin (Jeong and Puleo, 2009) and polyamines, appear as an advantageous alternative to the use of the expensive recombinant growth factors. Natural polyamines, such as spermidine, spermine and their precursors ornitine and putrescine, have been recently claimed to induce the differentiation of mesenchymal stem cells into osteoblasts (Helder and Wuisman, 2007; Tjabringa *et al.*, 2008), although their mechanisms of action are still not completely elucidated and the way of delivery is not established yet. The search for combining the osteoinductive capability of amine-bearing synthetic molecules with an affordable administration by minimally invasive techniques prompted us to explore the potential of block copolymers of the family of poloxamines. Poloxamines, commercially available under the trade name of Tetronic®, are X-shaped copolymers that bear four PEO-PPO arms connected to a central ethylenediamine group (Fig. 1). Differently from the natural polyamines that bear amine groups at one or both ends and the distance between amine groups inside the chain is of 3 or more carbon atoms, poloxamines only have two amine groups, which are separated by 2 carbon atoms and never placed at the end of the copolymer chain. The poloxamines has been exploited until now for rendering dually temperature and pH responsive micelles and gels (Alvarez-Lorenzo *et al.*, 2007, 2010a,b; Gonzalez-Lopez *et al.,* 2008, 2010; Chiappetta *et al.*, 2010), to favor the interactions with cells of tissue engineered-modular construct (Chiappetta and Sosnik, 2007) and to inhibit P-glycoprotein efflux (Alvarez-Lorenzo *et al.*, 2010a). The aim of this work was to elucidate the potential of poloxamines as the sole component of injectable scaffolds able to induce bone formation, but also as suitable carriers of rhBMP-2. The present study was organized in three main steps: (i) first, a cytocompatibility screening of poloxamine varieties covering a wide range of structural features (namely, molecular weight, length of the PEO and PPO blocks, and spatial arrangement of the blocks; Table 1) was carried out in order to identify the copolymers more suited to be in contact with mesenchymal stem cells and osteoblasts; (ii) second, the temperature-induced gelling, the incidence of autoclave sterilization on the viscoelastic properties of poloxamine solutions and their capability to control the delivery of a model protein (bovine serum albumin, BSA) were investigated; and (iii) finally, *in vitro* release

Table 1. Average molecular weight (BASF web page) and structural features of sequential ($(OE_{a/4}OP_{b/4})_2NCH_2CH_2N(OP_{b/4}OE_{a/4})_2$) and reverse ($(OP_{b/4}OE_{a/4})_2NCH_2CH_2N(OE_{a/4}OP_{b/4})_2$) poloxamine varieties, Tetronic® (Chiappetta and Sosnik, 2007), and of the poloxamer variety, Pluronic® F127 ($OE_{a/2}-OP_b-OE_{a/2}$) used as a control (Kabanov and Alakhov, 2002).

Copolymer	Molecular weight (Da)	Total EO units (a)	Total PO units (b)
Tetronic® 304	1650	15.0	17.1
Tetronic® 901	4700	10.7	72.9
Tetronic® 904	6700	60.9	69.3
Tetronic® 908	25000	454.5	86.2
Tetronic® 1107	15000	238.6	77.6
Tetronic® 1301	6800	15.5	105.5
Tetronic® 1307	18000	286.4	93.1
Tetronic® 150R1	7900	20	116.0
Pluronic® F127	12600	200	69

of rhBMP-2 and bioactivity and gene expression assays (cell differentiation to osteoblasts) were simultaneously carried out. Pluronic F127 was also included in the study as a control since this FDA-approved linear copolymer possesses certain structural similarities with poloxamines (namely, PEO and PPO blocks) and has been previously shown suitable as BMP-2 vehicle (Issa *et al.*, 2008). Selective staining to reveal differentiation to other lineages, namely adipocytes or chondrocytes, was also applied. The overall analysis of the information obtained may enable the relating of structural features of poloxamines with their osteoinductive ability and performance as BMP-2 carriers and should contribute to the rational design of optimized injectable scaffolds for tissue engineering.

Materials and Methods

Materials

Poloxamines Tetronic® 304, 901, 904, 908, 1107, 1301, 1307 and 150R1 (Table 1) and poloxamer Pluronic® F127 were supplied by BASF (Ludwigshafen, Germany); bovine serum albumin (BSA) and sodium azide by Fluka (now Sigma-Aldrich, St. Louis, MO, USA); rhBMP-2 (InductOs™ 12 mg, 1.5 mg/ml) by Wyeth Farma S.A. (now Pfizer, New York, NY, USA); kit ELISA Quantikine Immunoassay BMP-2 by R&D Systems (Minneapolis, MN, USA); alkaline phosphatase (ALP) substrate and p-nitrophenylphosphate solution by Sigma-Aldrich; kit BCA by Pierce (Rockford, IL, USA); and Cytotoxicity Detection Kit^PLUS (lactate dehydrogenase, LDH) by Roche (Basel, Switzerland). Balb/3T3 mouse fibroblasts clone A31 (CCL-163), CHO-K1 epithelial cells from ovary of chinese hamster (CCL-61) and SAOS-2 human osteogenic sarcoma cells (HTB-85) were purchased from the American Type Culture Collection (LGC Standards, Manassas VA). Mesenchymal stem cells StemPRO® human adipose-derived stem cells were supplied by Gibco (Invitrogen, Carlsbad, CA, USA). Dulbecco's Modified Eagle medium (D-MEM) Nutrient mixture F-12 Ham without phenol red was supplied by Sigma-Aldrich (USA) and MesenPRO RS™ medium (MesenPRO RS™ Basal Medium and MesenPRO RS™ Growth Supplement) by Gibco (Invitrogen). Fetal bovine serum (FBS) was supplied by BioWhittaker® Lonza (Basel, Switzerland),

gentamicine from B. Braun (Melsungen, Germany), β-glycerophosphate, dexamethasone and ascorbic acid by Sigma-Aldrich. Purified water was obtained by reverse osmosis (MilliQ®, Millipore, now Merck, Darmstadt, Germany). Other reagents were of analytical grade.

Cytocompatibility screening

Tetronic 304, 901, 904, 908, 1107, 1301, 1307 and 150R1 solutions (10% w/w) were prepared by dissolving the required amount of each polymer in phosphate buffer pH 7.4. The solutions were filtered by 0.2 μm membranes (Millex™, Millipore). Balb/3T3 and CHO-K1 cells were cultured in D-MEM /Nutrient mixture F-12 Ham without phenol red, containing 10% v/v FBS and gentamicine (0.1 mg/mL). 5,000 cells/well (50 μL medium) were seeded in 96-well plates and 50 μL of polymer solutions (conveniently diluted to 0.01, 0.1, 1 and 5%) were added. Final FBS concentration was 5% v/v. Negative and positive controls were carried out by replacing the polymer solutions with D-MEM solely or D-MEM containing a lysis factor (included in the Cytotoxicity Detection Kit^PLUS (LDH), Roche), respectively. Plates were incubated for one or three days at 37 ºC, in a 5% CO_2 humidified atmosphere. Cells survival was evaluated at 24 and 72 h by using the Cytotoxicity Detection Kit^PLUS (LDH) and the absorbance was measured at 490 nm using a microplate reader (BIORAD Model 680, Hercules, CA USA). Each experiment was carried out in triplicate and repeated twice.

Osteoblast viability

Tetronic 908, 1107, 1301 and 1307 solutions at 20% w/w were prepared in D-MEM without phenol red and filtered through 0.2 μm membranes (Millex™, Millipore). SAOS-2 cells were cultured in D-MEM supplemented with 10% w/v FBS and gentamicine (0.1 mg/mL). 200,000 cells/well (1.5 mL) were seeded in 24-well plate (area available for cell growth 1.9 cm²) and 0.5 mL polymer solution was added. Final FBS concentration was 7.5% v/v. Some plates were incubated at 37 ºC for 24 h and others for 72 h. Then, the cells were detached by addition of trypsin and incubated at 37 ºC for 5 min in a humidified atmosphere with 5% CO_2. After incubation, cells were centrifuged at 1,400 rpm for 4 min (Eppendorf centrifuge 415R, Eppendorf Ibérica, Madrid, Spain) and resuspended in fresh D-MEM. Aliquots (100 μL) of cell suspension diluted with phosphate buffer

pH 7.4 (200 μL) were transferred to a cytospin (Hettich Zentrifugen, Tuttlingen, Germany; Zytokammer 4x1 mL) and centrifuged in order to fix cells on a microscope slide. Finally, a calcein/propidium iodide staining (phosphate buffer solution: calcein: propidium iodide 98:1:1) was done to assess live/dead populations. Viability values were calculated using a light microscope (Optiphot2, Nikon, Tokyo, Japan) with green and red filters connected to image analysis software (Soft Imaging System GmbH, Münster, Germany; Version 3.2 Build 607, Germany). Each experiment was carried out in triplicate and repeated twice. Cell fraction cultured with Tetronic 1301 could not be harvested, mainly because the hydrophobicity of this copolymer caused it to remain partially adhered to the cells pellet, affecting the cell viability quantification. To overcome this drawback, 1.5 mL of Tetronic 1301 dispersion were placed into the upper compartment of 6-well plate Transwell® permeable supports and 800,000 cells/well (2.5 mL) were seeded in the lower compartment (area available for cell growth 9.5 cm^2). Plates were incubated for 24 h at 37 ºC in a 5% CO_2 humidified atmosphere and cell viability was determined as explained above. In any of the two osteoblast viability experiments described in this section, the number of SAOS-2 cells was chosen to be close to 100,000 cells per square centimeter of well, as recommended by the manufacturer of the plates.

Oscillatory rheology of poloxamine solutions
The storage or elastic (G´) and the loss or viscous (G´´) moduli of 20% w/w Tetronic 908, 1107, 1301 and 1307 solutions in phosphate buffer pH 7.4 and D-MEM were evaluated in triplicate, before and after being autoclaved (121 ºC, 20 min, Raypa AES-12, Barcelona, Spain), using a Rheolyst AR-1000N rheometer equipped with an AR2500 data analyzer, a Peltier plate and a cone geometry (6 cm diameter, 2.1º) (TA Instruments, Newcastle, UK). To determine the influence of temperature on both moduli, tests were carried out at 5 rad/s from 15 ºC to 45 ºC with a heating rate of 2 ºC/min. Evaporation was prevented using silicon oil as solvent trap.

BSA release test
BSA (50 mg) was added to 20% w/w Tetronic 908, 1107, 1301 and 1307 solutions in phosphate buffer pH 7.4 (8 mL). 2 mL aliquots of each dispersion were placed into test tubes (13.5 mm inner diameter) at 37 ºC. Then, 5 mL of phosphate buffer pH 7.4 at 37 ºC were slowly added on the gel phase. The system was kept at 37 ºC, under oscillating agitation (50 osc/min), for several weeks. The surface area of the gel in contact with the release medium was 143.1 mm^2. Samples of the supernatant (1 mL) were taken at pre-established time intervals and replaced with equal volume of buffer at 37 ºC. BSA concentration was spectrophotometrically determined at 278 nm (Agilent 5348, Santa Clara, CA, USA). The experiments were carried out in triplicate.

rhBMP-2 release test
Tetronic 908, 1107 and 1307 and Pluronic F127 at 26.5% w/w solutions in phosphate buffer pH 7.4 were autoclaved (121 ºC, 20 min, Raypa AES-12, Barcelona, Spain) and

then mixed with rhBMP-2 solution (0.15 mg/mL). Final copolymer and rhBMP-2 concentrations were 20% and 0.037 mg/mL, respectively. 1 mL aliquots of each dispersion were placed into test tubes (13.5 mm inner diameter) at 37 ºC. Negative controls (copolymer without rhBMP-2) were also tested. Then, 5 mL of phosphate buffer pH 7.4 with 0.02% w/v sodium azide at 37 ºC were slowly added on the gel phase. The system was kept at 37 ºC under oscillating agitation (50 osc/min). The surface area of the gel in contact with the release medium was 143.1 mm^2. Samples of the supernatant (1 mL) were taken at pre-established time intervals, divided into two aliquots (0.5 mL) and frozen at -20 ºC. Each extracted sample was immediately replaced by 1 mL buffer at 37 ºC. rhBMP-2 concentration in the release medium was quantified after 1:50,000 dilution, using a sandwich enzyme immunoassay technique (Kit ELISA Quantikine Immunoassay BMP-2, R&D Systems). The absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm, was measured using an ELISA reader (BIORAD Model 680 Microplate Reader). Quantification interval ranged from 62.5 pg/mL to 4,000 pg/mL. Each experiment was carried out in duplicate and repeated twice.

Bioactivity of poloxamine gel formulations: ALP assay and histochemical analyses
Mesenchymal stem cells were cultured in MesenPRO RS™ medium (Gibco, Invitrogen) supplemented with 1% glutamine and 1% antimicrobial agents (penicillin, streptomycin, and amphotericin B) and seeded (3x10^4 cells/well, 2.5 mL) in 6-well plates. 200 μL of copolymer dispersion (20% w/w) containing or not rhBMP-2 (0.037 mg/ml) were placed into the upper compartment of 6-well plate Transwell® permeable supports. Thus, the amounts of copolymer (poloxamine or poloxamer) and rhBMP-2 added to each well were 40 mg and 7.4 μg, respectively. Negative and positive controls were carried out with cells in culture medium (neither copolymer nor rhBMP-2 added) and cells in osteogenic differentiation medium (10 mM β-glycerophosphate, 100 nM dexametasone and 50 μM ascorbic acid in the culture medium), respectively. Plates were incubated at 37 ºC, in a humidified atmosphere with 5% CO_2. The medium was replaced twice a week. Cells were observed at inverted microscopy (10x) to discard possible contamination by bacteria. Each experiment was carried out in triplicate and repeated twice.

To carry out the alkaline phosphatase (ALP) assay, cells were lysed at 3, 7, 14 and 23 d by addition of 150 μL Tris HCl buffer 10 mM pH 7.5 with 0.1% Triton X-100. Samples were exposed to 3 freezing (-80 ºC)/thawing cycles (45 min per cycle). Lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4 ºC. The samples (50 μL) were incubated with ALP substrate (150 μL) in 96-well plates at 37 ºC for 30 min. The absorbance was read at 405 nm using an ELISA plate reader (BIORAD Model 680 Microplate Reader). A p-nitrophenylphosphate calibration curve was used to determine ALP concentration. Each ALP activity measurement was normalized by the protein content, which was measured by the BCA protein assay (Kim and Valentini, 2002). Results were reported as nanomoles ALP/min/mg protein.

Various histochemical analyses were carried out in triplicate. ALP activity was visualized at 14 d as follows. The cells were fixed using 4% paraformaldehyde solution for 5 min, washed with phosphate buffer, incubated in darkness with 0.1% naphthol ASMX-phosphate and 0.1% fast violet in 56 mM AMPD (2-amino-2-methyl propanediol) and observed by light microscopy. Alizarin red staining was used to detect the mineralization of the matrix at 14 and 23 d. Cells were fixed with cold ethanol:water 70:30 v/v for 1 h, washed with water twice, stained with 2% alizarin red for 30 min, washed with water three times and observed by microscopy. Oil Red O staining was performed at 7, 14 and 23 d, as described by the manufacturer (SC Protocol 00011, Thermo Scientific, Logan, UT, USA), to detect adipogenic activity and mature adipocytes. Chondrogenic differentiation of mesenchymal stem cells was observed using Alcian blue staining. Briefly, after 7, 14 and 23 d in contact with the copolymer gels, the medium was removed and the cells were rinsed once with PBS and fixed with 4% formaldehyde solution for 30 min. Then, the wells were rinsed with PBS and 1% Alcian blue solution in 0.1 N HCl was added and kept for 30 min. The wells were again rinsed three times with 0.1 N HCl and then with water, and the cells were visualized by light microscopy to search for the blue staining indicative of the synthesis of proteoglycans by chondrocytes.

Cell proliferation

Mesenchymal stem cells were cultured as described above in the presence of copolymer dispersions (20% w/w) containing or not containing rhBMP-2 (0.037 mg/mL). Cell proliferation was assessed by MTT assay (Roche). At 4, 7, 14 and 23 d of culture, Transwell® compartments were retired and 1.5 mL of culture medium withdrawn. Then, 100 μL MTT solution (5 mg/mL stock in PBS) were added to each well containing 1 mL of remaining culture medium and the plates were incubated during 4 h at 37 ºC. Then 1 mL MTT solvent (10% SDS in 0.01 M HCl) was added and incubated overnight at 37 ºC. Aliquots of wells were removed, kept in Eppendorf tubes and frozen at -80 ºC until analysis. Tubes were centrifuged at 13,000 rpm for 5 min, the supernatants were transferred to 96-well plates and the absorbance was recorded using an ELISA plate reader at 550 nm (BIORAD Model 680 Microplate Reader, USA). Cell number was estimated from a calibration curve constructed using cultures with known number of cells.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Mesenchymal stem cells were cultured as described in the *Bioactivity of poloxamine gel formulations* section and total cellular RNA was extracted at days 7, 10 and 14 using TRIzol® (Invitrogen) according to the manufacturer's protocol. Phase separation was carried out using chloroform and the resultant RNA pellets were washed with ethanol:water 70:30 v/v, dried under air and resuspended in DEPC-treated water. The absorbance of RNA systems was measured at 260 nm using a biophotometer (Eppendorf, Hamburg, Germany) and RNA concentration was calculated. RNA (5 μg) was reversed transcribed into cDNA using Maloney murine leukemia virus (M-MLV) reverse transcriptase and random hexamers (Invitrogen). Reverse transcription (RT) conditions were 37 ºC for 50 min, 42 ºC for 15 min and 95 ºC for 5 min. To assess mRNA expression, a semiquantitative RT-PCR method was used by measuring PCR products during the exponential phase of cDNA amplification. The generated cDNA was amplified by using primers for CBFA-1, ALP, and collagen type I (Li *et al.*, 2009). Glyceraldehide-3-phosphate dehydrogenase (GAPDH) was used as a control for assessing PCR efficiency (Li *et al.*, 2009). The PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide. Primers sets and PCR products sizes are listed in Table 2.

Statistical analysis

ALP activity registered for the different copolymers and formulations was compared using ANOVA and the Fisher´s Least Significant Differences (LSD) multiple range test (Statgraphics Plus for Windows 5.1, Statistical Graphics Corp., Warrenton, VA, USA).

Results

Cytocompatibility screening

A preliminary screening of poloxamine varieties regarding their cytocompatibility against Balb/3T3 fibroblasts and CHO-K1 epithelial-like cells was carried out according to the UNE-EN ISO 10993-5 protocol, although the assay time was not limited to 24 h but also extended to 72 h. Tetronic 908, 1107, 1301, 1307 and 150R1 were found to be the most cytocompatible poloxamines, showing high

Table 2. Primers sets and PCR products sizes.

Gene names	Primers sequences	Product size	Annealing temperature
CBFA-1 (Runx2)	F: 5´CAGACCAGCAGCACTCCATA-3´ R :5´-TCCAATATGGTCGCCAAACA-3´	256	52
ALP	F: 5´-CCCAAAGGCTTCTTCTTG-3´ R: 5-CTGGTAGTTGTTGTGAGCAT-3´	356	55
Collagen type I	F: 5´-CGCCATCAAGGTCTACTGC-3´ R: 5´-GAATCCATCGGTCATGCTCT-3´	148	54
GAPDH	F: 5´-ACCACAGTCCATGCCATCAC-3´ R: 5´-TCCACCACCCTGTTGCTGTA-3´	452	55
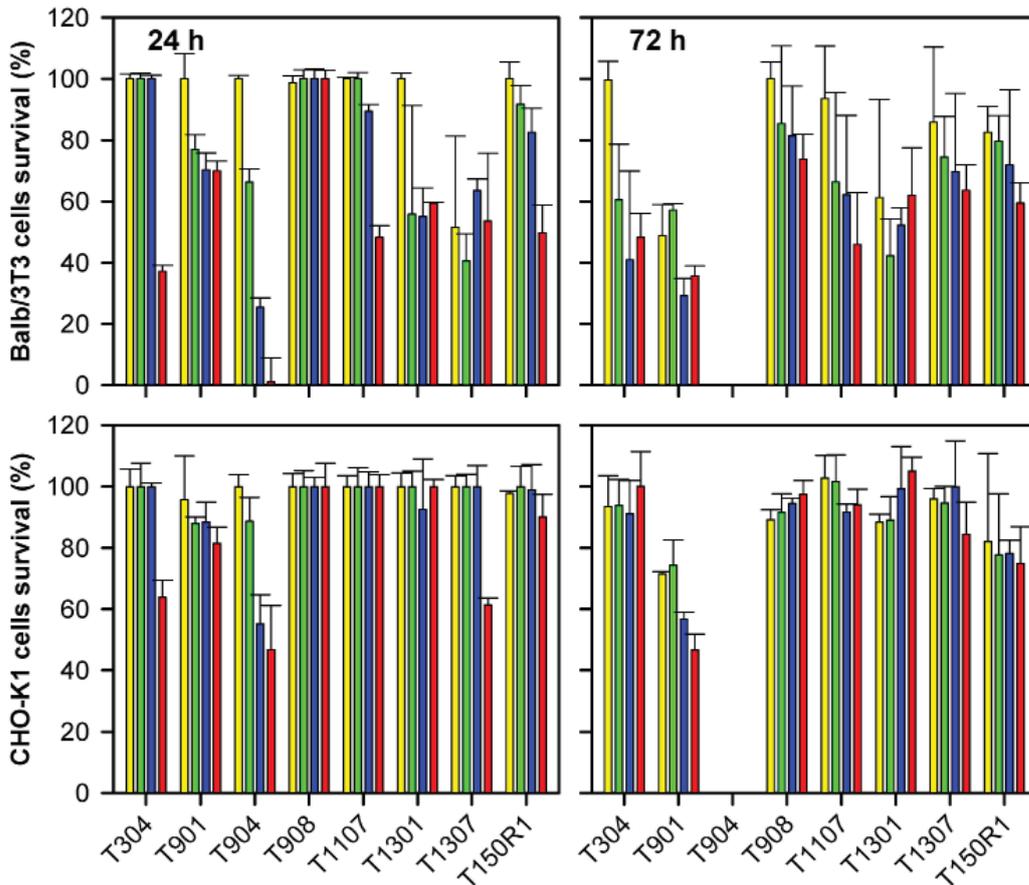
Fig. 2. Cell viability after 24 and 72 h in contact with poloxamines at 0.01% w/w (yellow columns), 0.1% w/w (green columns), 1% w/w (blue columns) and 5% w/w (red columns). All tests were done in triplicate and the standard deviations are shown as error bars.
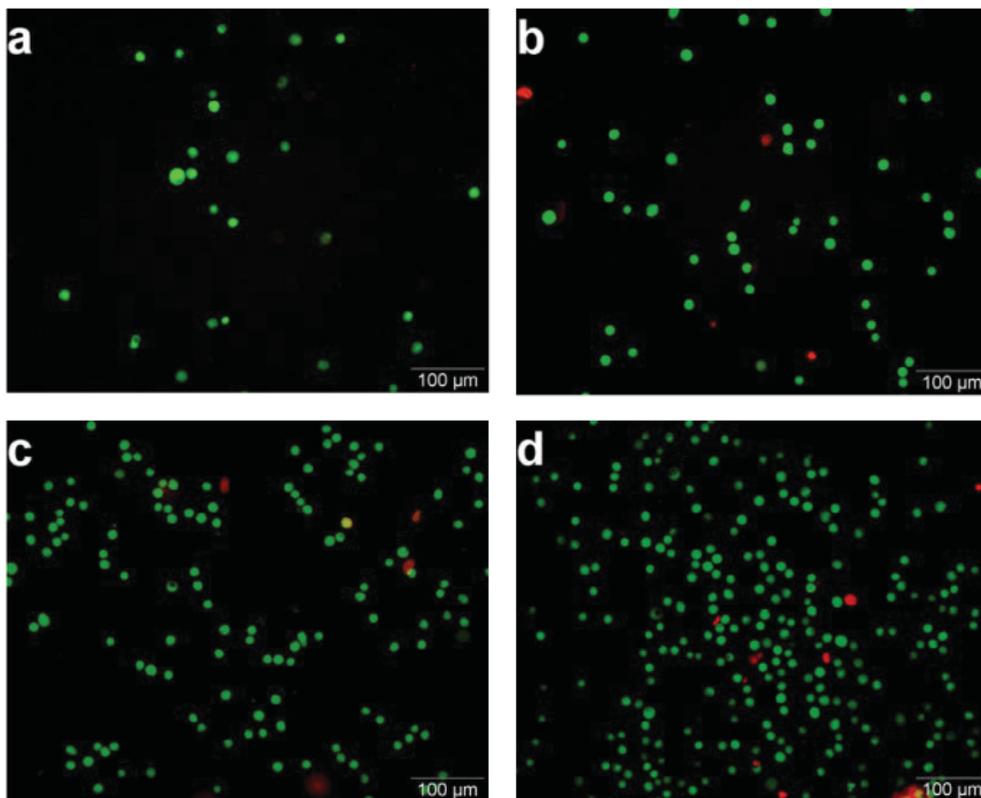
Fig. 3. Viability of SAOS-2 cells after 24 h in contact with 20% w/w Tetronic 908 (**a**; 96.7% viability), Tetronic 1107 (**b**; 90.3 %), Tetronic 1301 (**c**, 95.3 %) and Tetronic 1307 (**d**; 95.8 %). The live cells stained with calcein AM appear in green and the dead cells stained with propidium iodide appear in red.
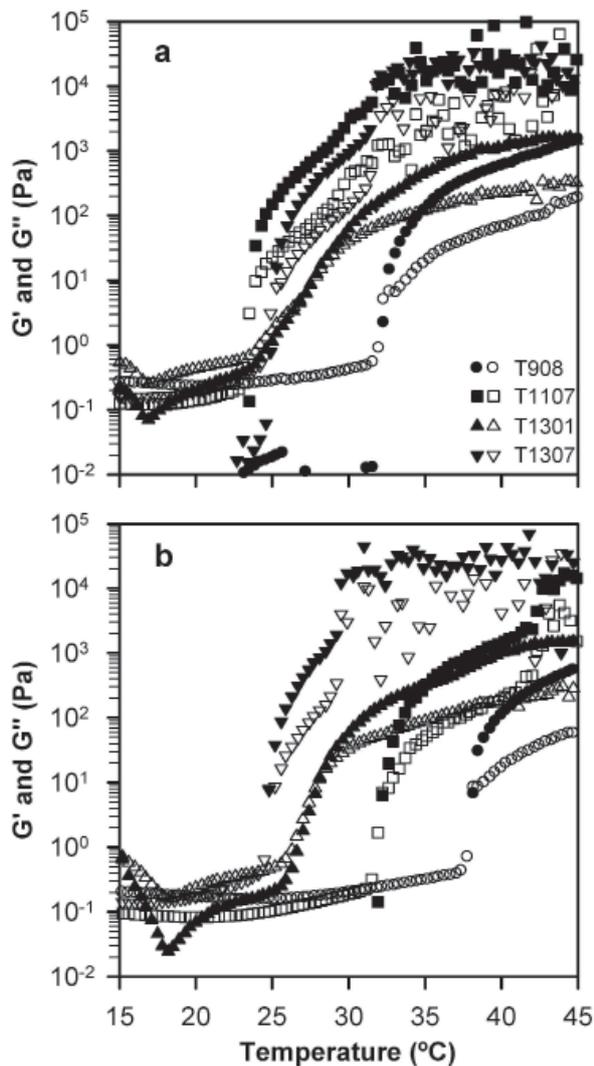
Fig. 4. Temperature dependence of the storage (G', solid symbols) and loss (G'', open symbols) moduli of 20% w/w poloxamine solutions in **(a)** phosphate buffer pH 7.4 and **(b)** DMEM after being autoclaved.

viability levels after 24 or 72 h in contact with the cells (Fig. 2). Correspondingly, Tetronic 901 and 904 were the less cytocompatible, particularly when tested against Balb/3T3 cells. No cell survival was observed after being in contact with Tetronic 904 for 72 h. Cytotoxicity experiments carried with Pluronic F127 revealed 100% cell survival at 24 h. Balb/3T3 fibroblasts viability at 72 h was 100% for 0.01% Pluronic, but decreased up to 55% for 5% Pluronic. CHO-K1 cells viability at 72 h ranged from 68 to 46% as the Pluronic F127 concentration increased from 0.01 to 5%.

The viability of SAOS-2 cells (human osteoblast cell line) after 24 h in the presence of 20% w/w Tetronic 908, 1107, 1301 and 1307 medium was above 90% as observed applying the live/dead staining (Fig. 3). It should be noticed that, since Tetronic 1301 is quite hydrophobic, the copolymer remained attached to the cell pellet and an alternative approach had to be implemented to evaluate cytocompatibility. Such an approach consisted in pouring the copolymer solution on the Transwell membrane above the cells inside the well. The number of cells per unit of surface was kept constant in all experiments with SAOS-
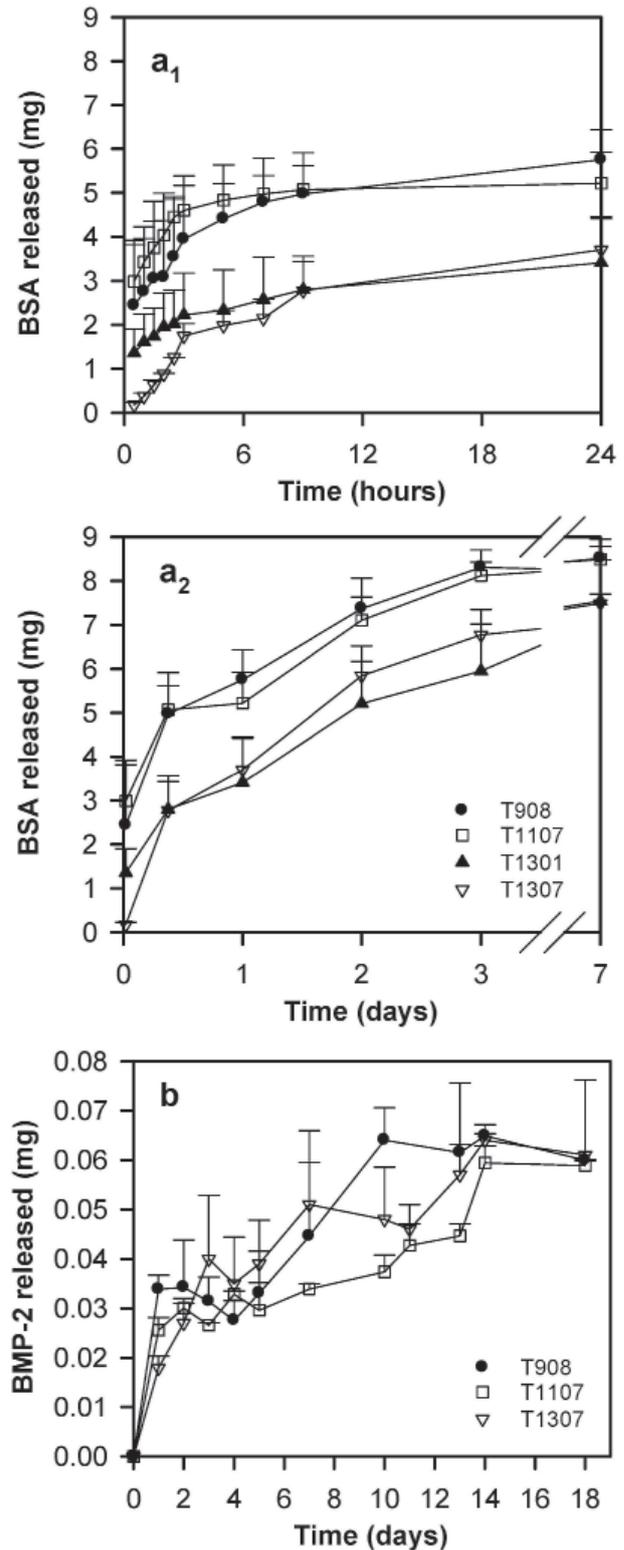
Fig. 5. BSA (**a₁**, up to 24 h, and **a₂**, up to 7 d) and rhBMP-2 **(b)** release profiles from 20% w/w poloxamine gels in phosphate buffer pH 7.4 at 37 ºC. In the case of rhBMP-2, the release medium was supplemented with sodium azide (0.02% w/v). The data plots only show one standard deviation in order to reduce overlapping.
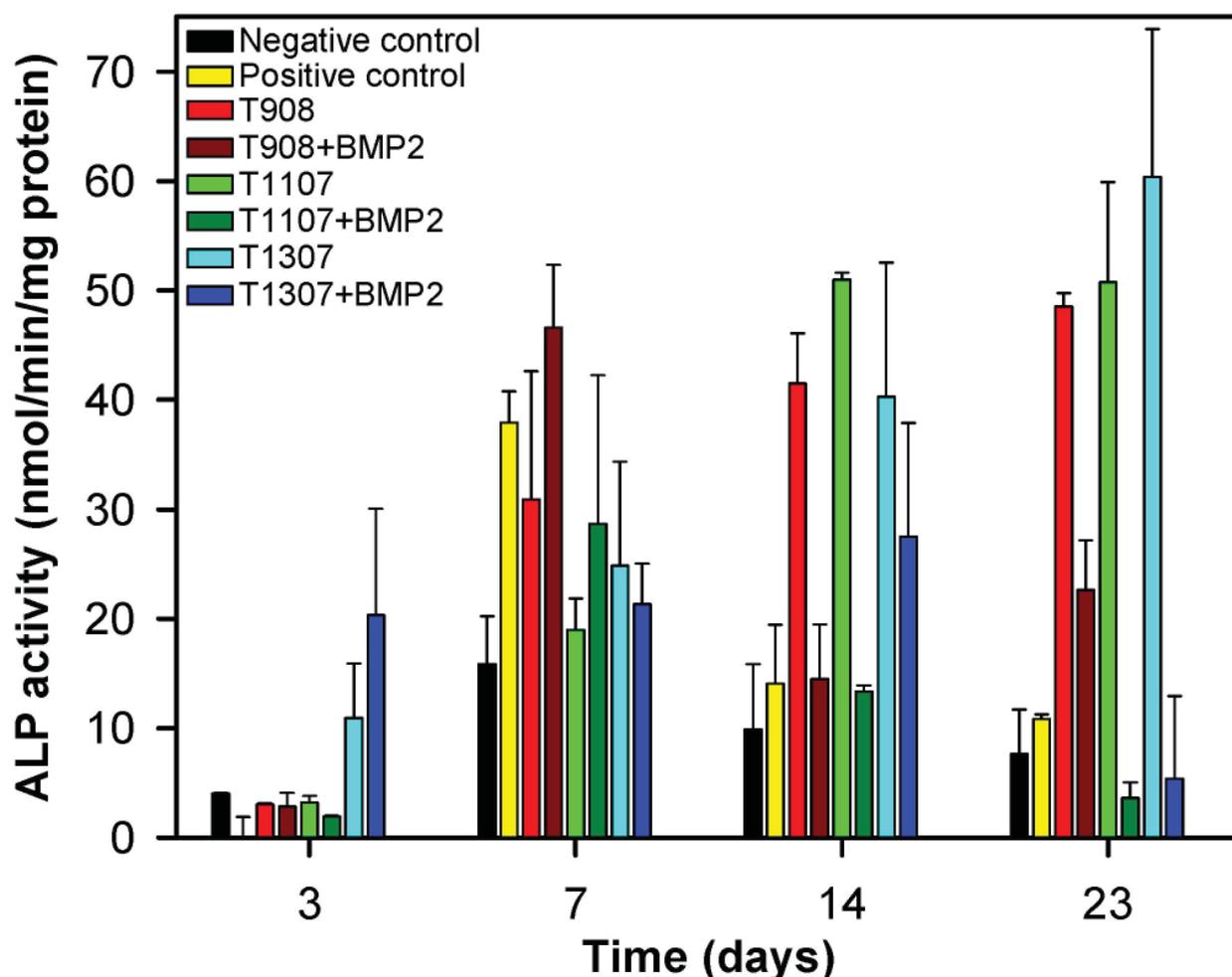
Fig. 6. Stimulation of ALP activity in mesenchymal stem cells induced by rhBMP-2-loaded poloxamine gels or by poloxamine solely gels. Bioactivity of positive (standard osteoinductive medium) and negative (cells alone) controls are also shown. Data are shown as mean + S.D.; *n*= 4.

2, but the relative amounts of cells, polymer, and medium were increased 4, 1.67, and 3-fold in the Tetronic 1301 experiments compared to those carried out with Tetronic 908, 1107 and 1307. Volume restrictions in the 6-well plate Transwell® prevented the use of a greater volume of Tetronic 1301 solution and medium.

Sol-to-gel phase transitions
The dependence of viscoelastic parameters on temperature of 20% w/w poloxamine in phosphate buffer pH 7.4 and in D-MEM culture medium is shown in Fig. 4 a and b, respectively. At room temperature, Tetronic 908, 1107 and 1307 systems only exhibited low G'' modulus, with values below 1 Pa. Tetronic 1301 was the only with recordable G' values, but as observed for the other poloxamines G' and G'' moduli were below 1 Pa. As temperature increased, a sudden increase in both moduli occurred. The gel temperature (at which the G' and G'' crossover occurred) in phosphate buffer pH 7.4 was around 25 ºC for Tetronic 1107, 1301 and 1307, and 33 ºC for Tetronic 908 (Fig. 4a). Slightly greater gel temperature values were recorded for poloxamine dispersions prepared in D-MEM culture medium (Fig. 4b). Above the gel temperature and up to 45 ºC, the moduli slightly increased or reached a plateau,

which is characteristic of well-structured networks. G' values in that region ranged between 10^3 and 10^5 Pa, as observed for 20% w/w Pluronic F127 systems. Poloxamine dispersions were evaluated again after being autoclaved and the rheological profiles (data not shown) were superimposable upon those obtained before autoclaving.

BSA release
Poloxamine systems (20 % w/w) containing BSA were placed in test tubes, thermostatically controlled at 37 ºC, and when the temperature was stabilized, phosphate buffer pH 7.4 (also at 37 ºC) was slowly deposited on the surface of formulations. During the first three days of the experiment, two well differentiated phases (i.e., the gel at the bottom and the buffer on it) were clearly observed. After that time, the gel mixed with the medium and eventually became diluted in the buffer. Formulations containing Tetronic 908 or 1107 released 50% BSA in the first 5 h, while those prepared with Tetronic 1301 and 1307 released around 25% BSA in the same time (Fig. 5a$_1$). Then, the release was sustained up to 7 d at an almost constant rate (Fig. 5a$_2$). Tests carried out using poloxamine dispersions at greater concentration (25% w/w) did not reveal relevant changes in BSA release profiles.
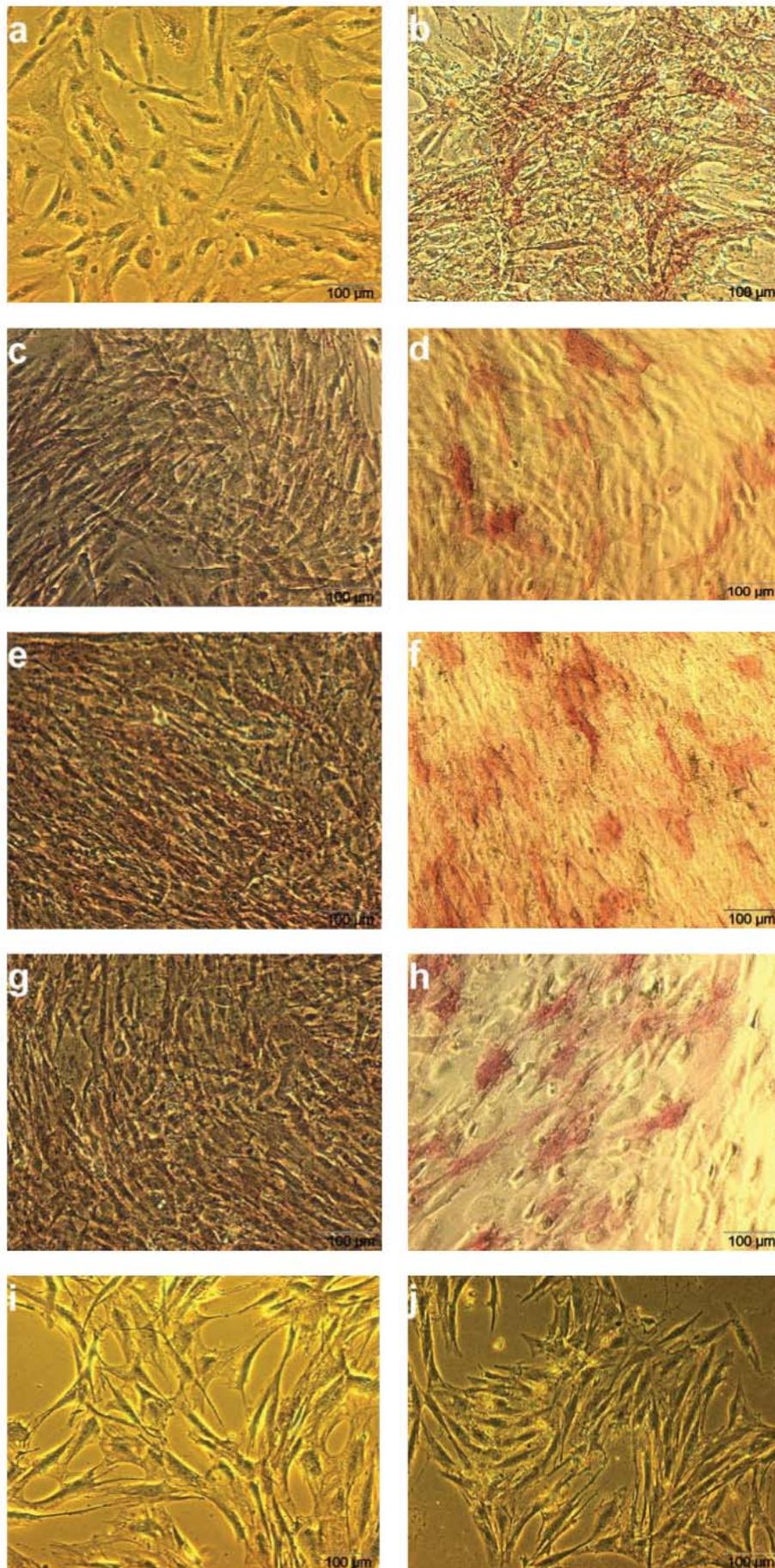
Fig. 7. ALP staining of cells at 14 days in control medium **(a)**, osteogenic medium **(b)**, and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, Tetronic 1307 solely **(g)** and with rhBMP-2 **(h)**, and Pluronic F127 solely **(i)** and with rhBMP-2 **(j)**.
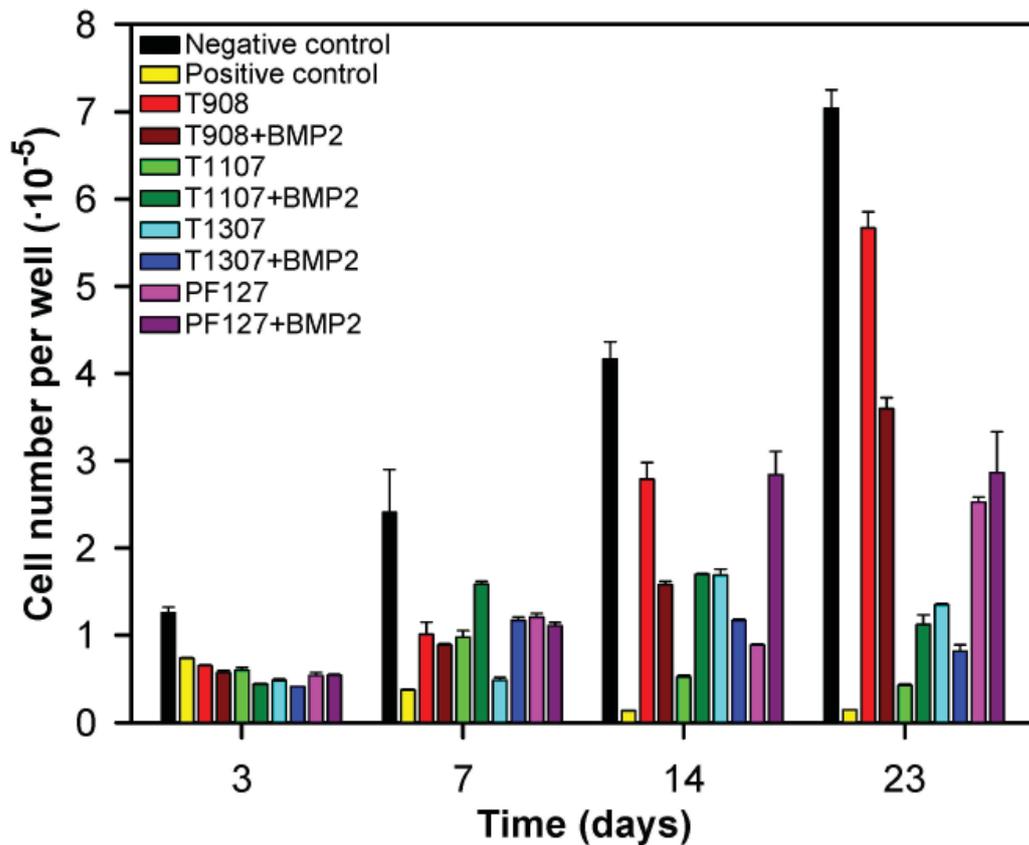
Fig. 8. Time-evolution of cell proliferation (determined using MTT assay and mesenchymal stem cells) in MesenPRO RS™ culture medium (negative control), osteogenic medium (positive control) and in the presence of poloxamine or poloxamer gels solely or loaded with rhBMP-2.

rhBMP-2 release and bioactivity tests

Commercial rhBMP-2 solution was readily mixed with concentrated poloxamine dispersions to render a rhBMP-2 concentration of 0.037 mg/mL in 20% w/w poloxamine solutions. Similarly prepared formulations were used for the *in vitro* release tests, bioactivity and gene expression assays. Since Tetronic 1301-based gels exhibited phase separation from the culture medium during the bioactivity assay, these formulations were discarded for further studies. The release experiments were carried out in phosphate buffer containing sodium azide (0.02% w/v). Active rhBMP-2 was specifically quantified using a sandwich enzyme immunoassay technique (Quantikine BMP-2 Immunoassay, R&D Systems USA). rhBMP-2 *in vitro* release profiles are shown in Fig. 5b. As observed for BSA release profiles, a fast delivery of rhBMP-2 occurred in the first day (amount released ranked in the order Tetronic 908 > 1107 > 1307) followed by a slower release for two weeks (100% release was achieved).

The bioactivity of rhBMP-2 released from poloxamine gels was assessed by monitoring the ALP activity of mesenchymal stem cells (Fig. 6). Pluronic F127 was also tested at the same concentration as the poloxamines. Negative (cells in medium without additives) and positive (cells in osteogenic differentiation medium) control assays were carried out as well. Negative controls revealed that the ALP activity of mesenchymal cells slightly increased at day 7, returning then to basal values. The ALP activity

of the cells cultured in the osteogenic medium (positive control) showed a maximum at day 7, which was much more intense than that observed for the negative control. ANOVA test of ALP activity at day 7 showed significant differences when all systems were compared ($F_{8,9 d.f.}$ = 5.27; $\alpha < 0.05$). LSD tests indicated that the ALP activity of Pluronic F127 solely, Tetronic 1107 solely and Tetronic 1307 with rhBMP-2 was no different from that of negative control.

Cells cultured with Tetronic 908 and 1107 gels loaded with rhBMP-2 exhibited an ALP activity pattern that resembled that obtained with the osteogenic medium. By contrast, the ALP activity of cells cultured with Tetronic 1307 gel loaded with rhBMP-2 initiated at day 3, remained practically constant up to day 14, and then decreased (Fig. 6). The highest ALP activity at day 7 was obtained for Tetronic 908 gels with or without rhBMP-2. No statistically significant differences between these gels and the positive control were found.

Poloxamine gels by themselves (without rhBMP-2) induced a progressive increase in ALP activity up to day 14 or latter, rendering ALP activity values well above those observed for the positive control. The ALP activities of Tetronic 1307 with and without rhBMP-2 at day 14 were not statistically different (LSD test after ANOVA of all systems, $F_{8,9 d.f.}$ = 12.08; $\alpha < 0.001$). At day 23, only cells cultured with poloxamines solely showed intense ALP activity; statistical test (LSD after ANOVA of all systems,
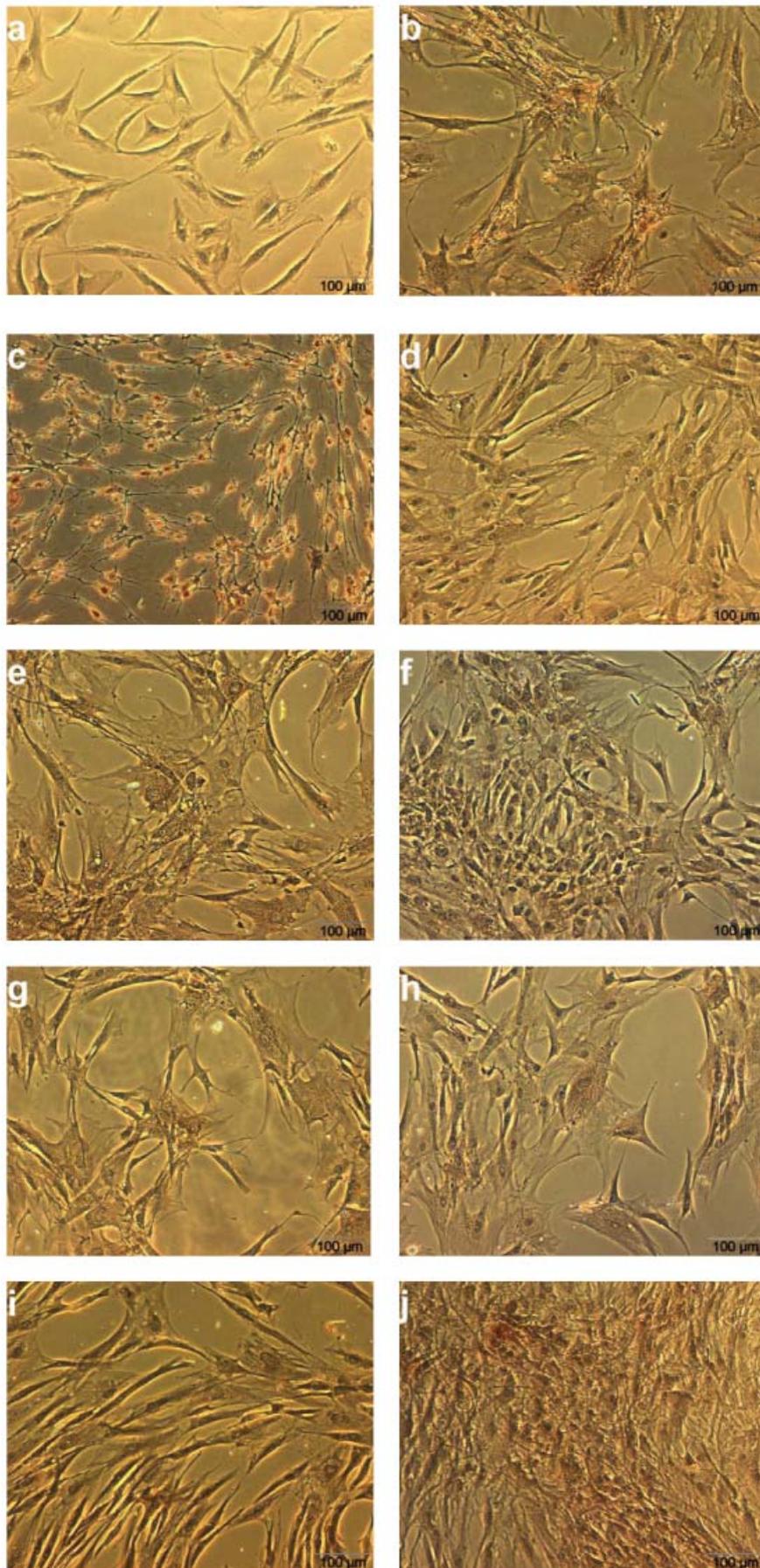
Fig. 9. Alizarin red staining of cells at 23 d in control medium **(a)**, osteogenic medium **(b)**, and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, Tetronic 1307 solely **(g)** and with rhBMP-2 **(h)**, and Pluronic F127 solely **(i)** and with rhBMP-2 **(j)**.
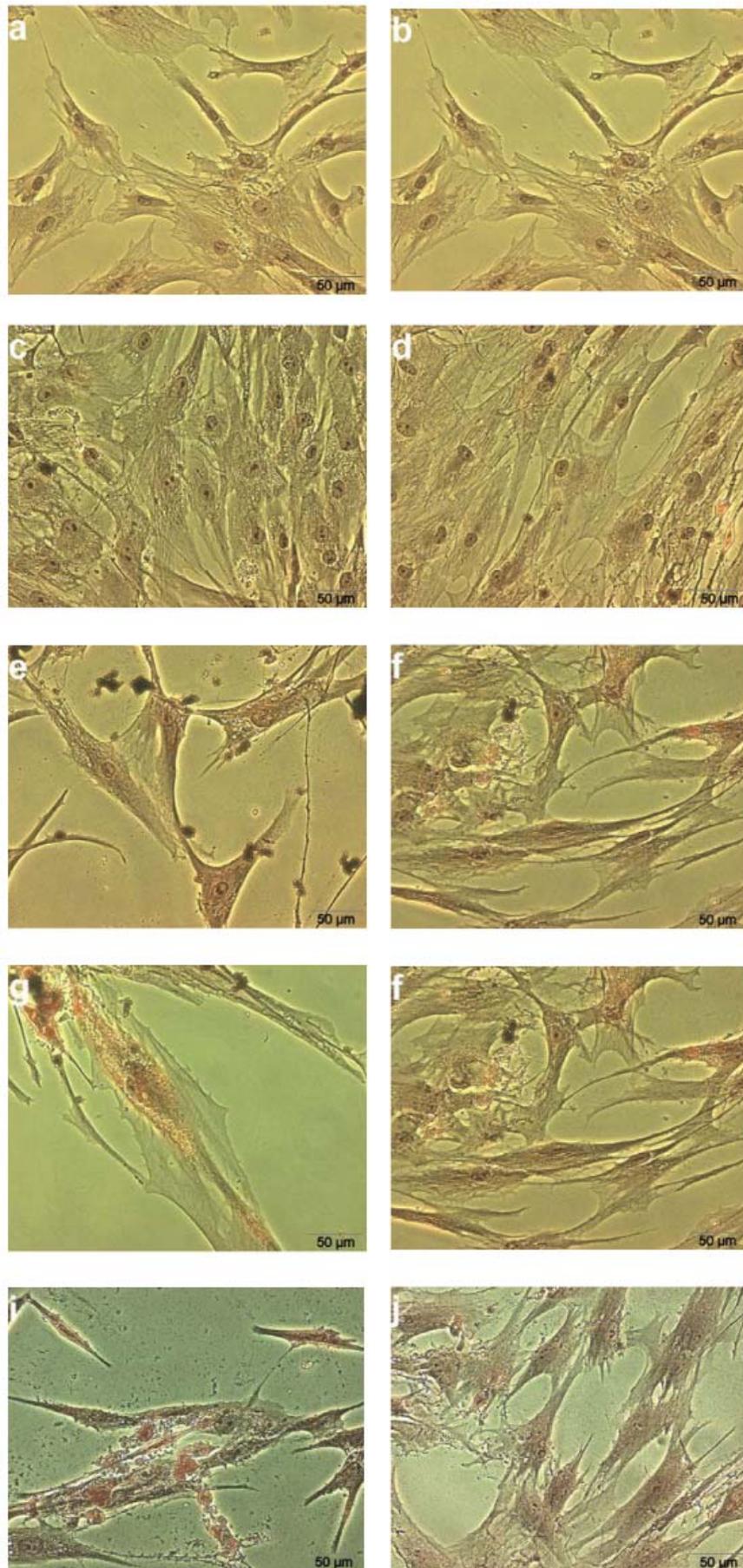
Fig. 10. Oil red staining of cells at 14 d with negative control **(a,b)**, and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, Tetronic 1307 solely **(g)** and with rhBMP-2 **(h)**, and Pluronic F127 solely **(i)** and with rhBMP-2 **(j)**.
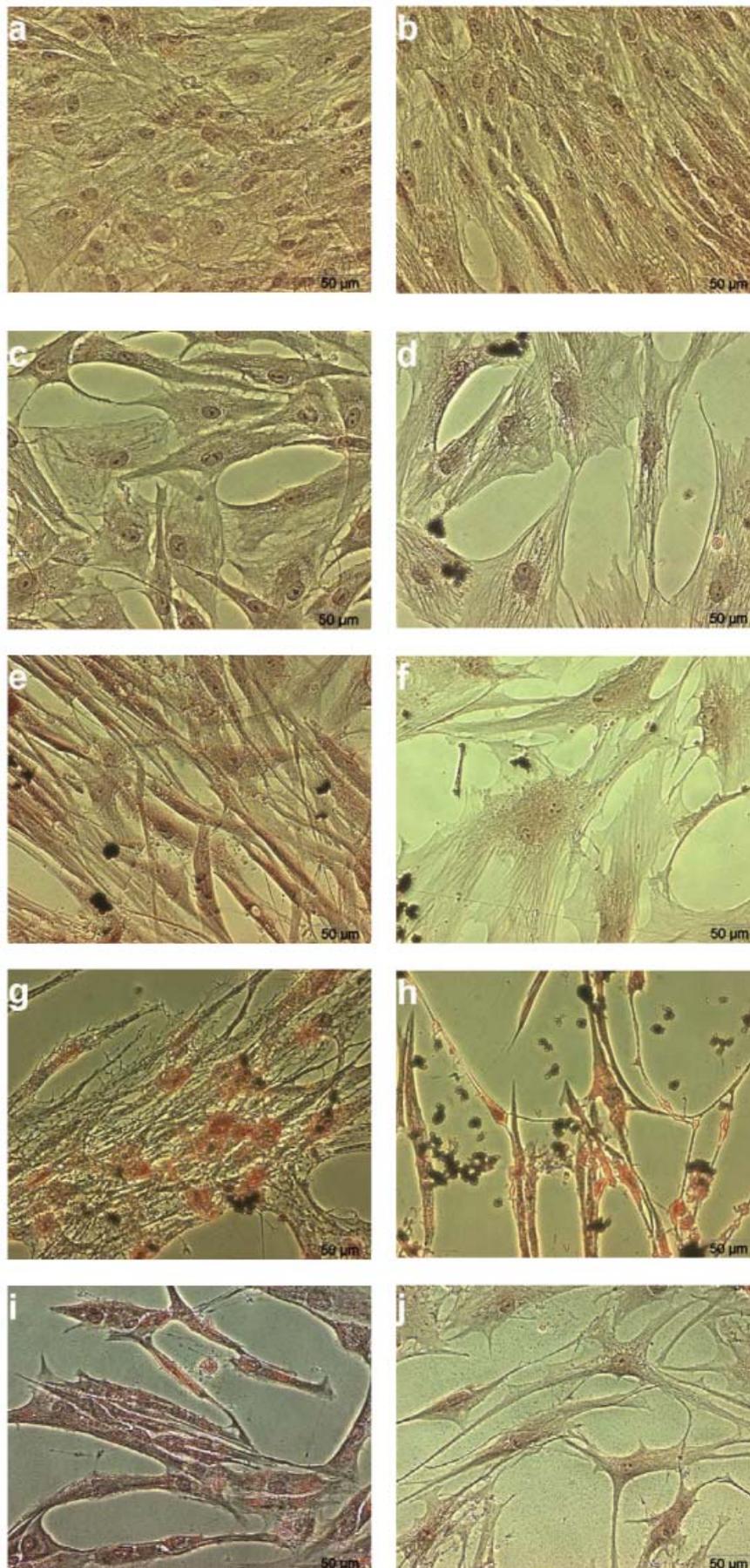
Fig. 11. Oil red staining of cells at 23 d with negative control **(a,b)**, and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, Tetronic 1307 **(g)** solely and with rhBMP-2 **(h)**, and Pluronic F127 solely **(i)** and with rhBMP-2 **(j)**.
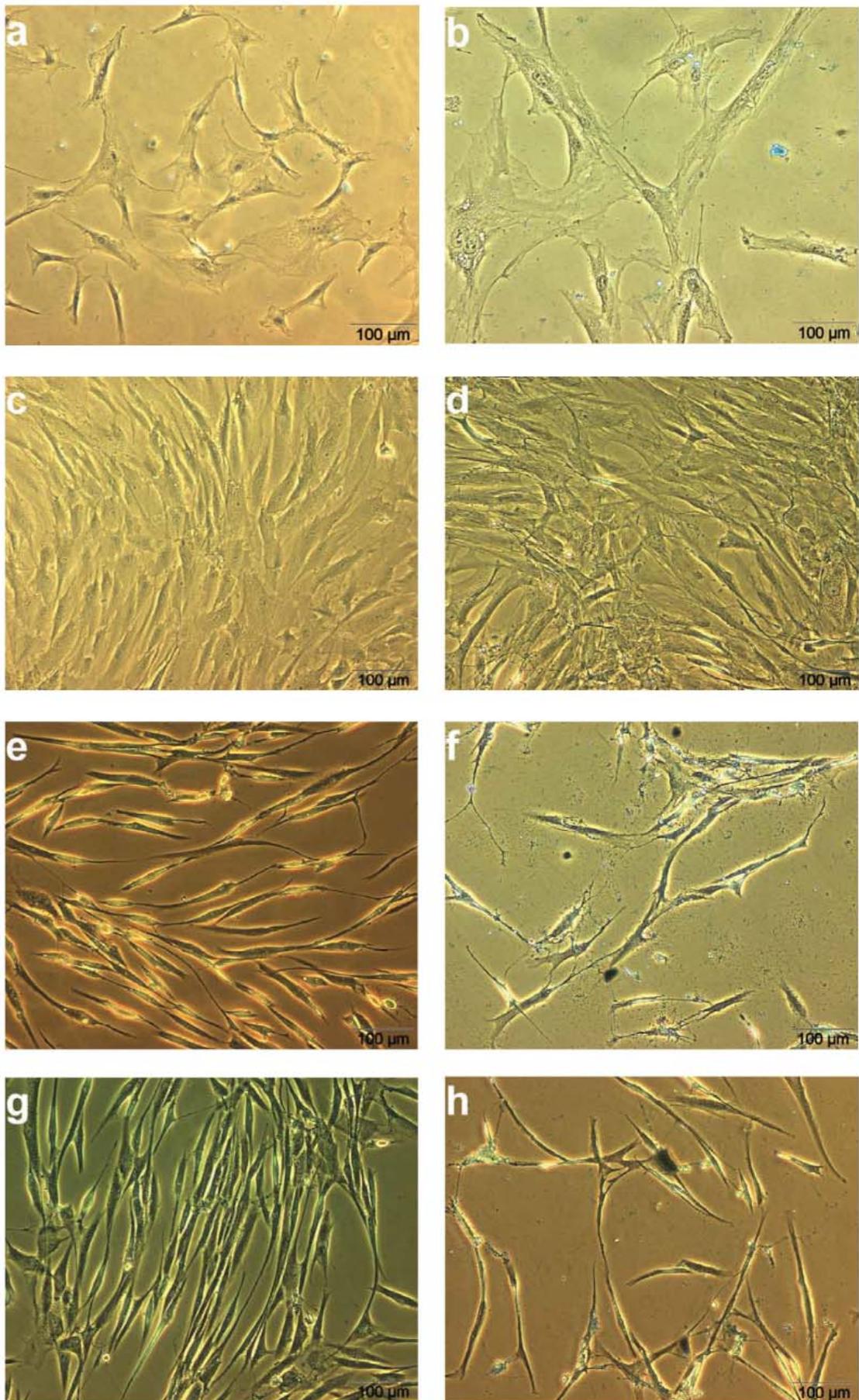
Fig. 12. Alcian blue staining of cells at 14 d with negative control **(a,b)**, and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, and Tetronic 1307 **(g)** solely and with rhBMP-2 **(h)**.
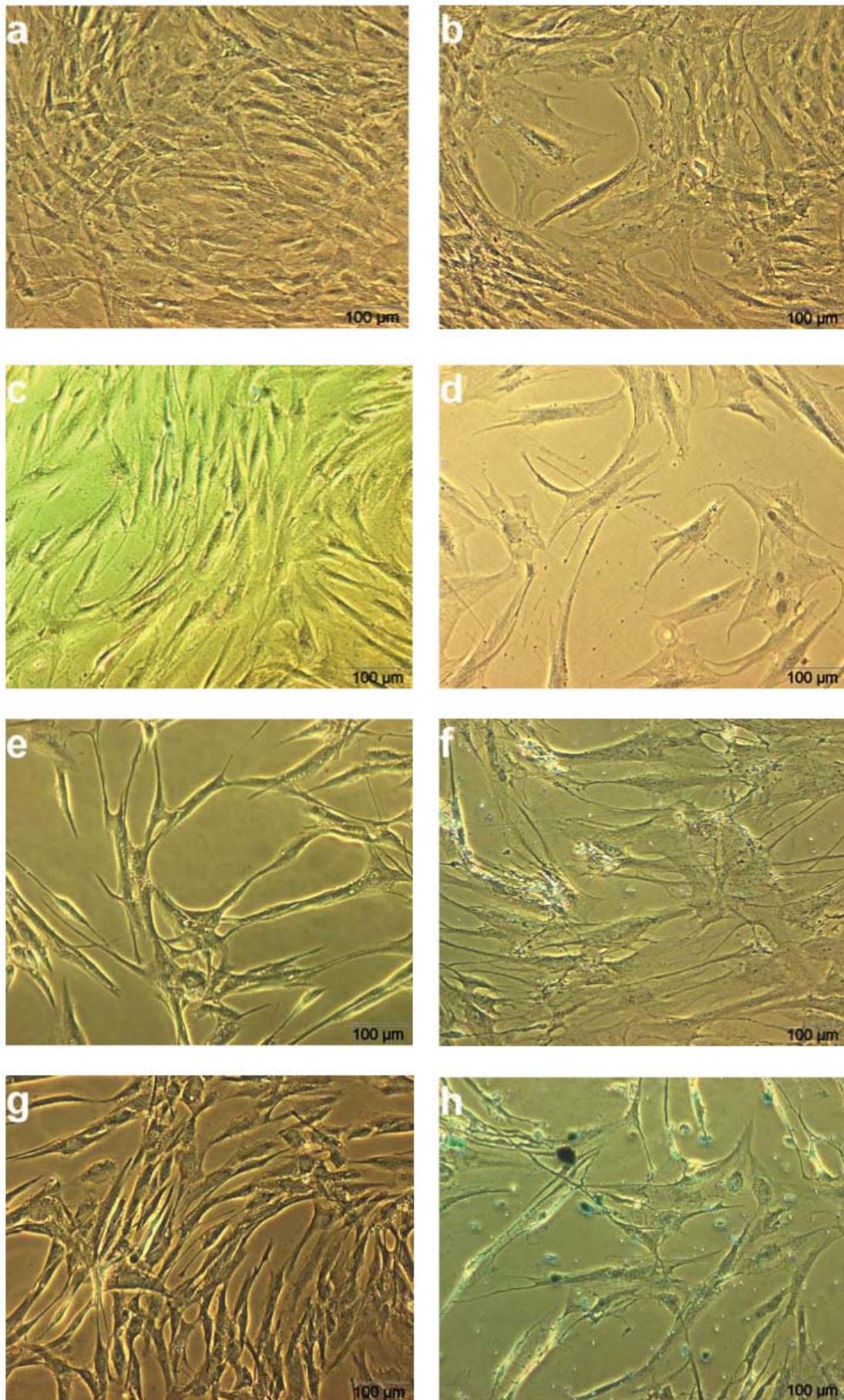
Fig. 13. Alcian blue staining of cells at 23 d with negative control (a,b), and in the presence of gels of Tetronic 908 solely **(c)** and with rhBMP-2 **(d)**, Tetronic 1107 solely **(e)** and with rhBMP-2 **(f)**, and Tetronic 1307 solely **(g)** and with rhBMP-2 **(h)**.
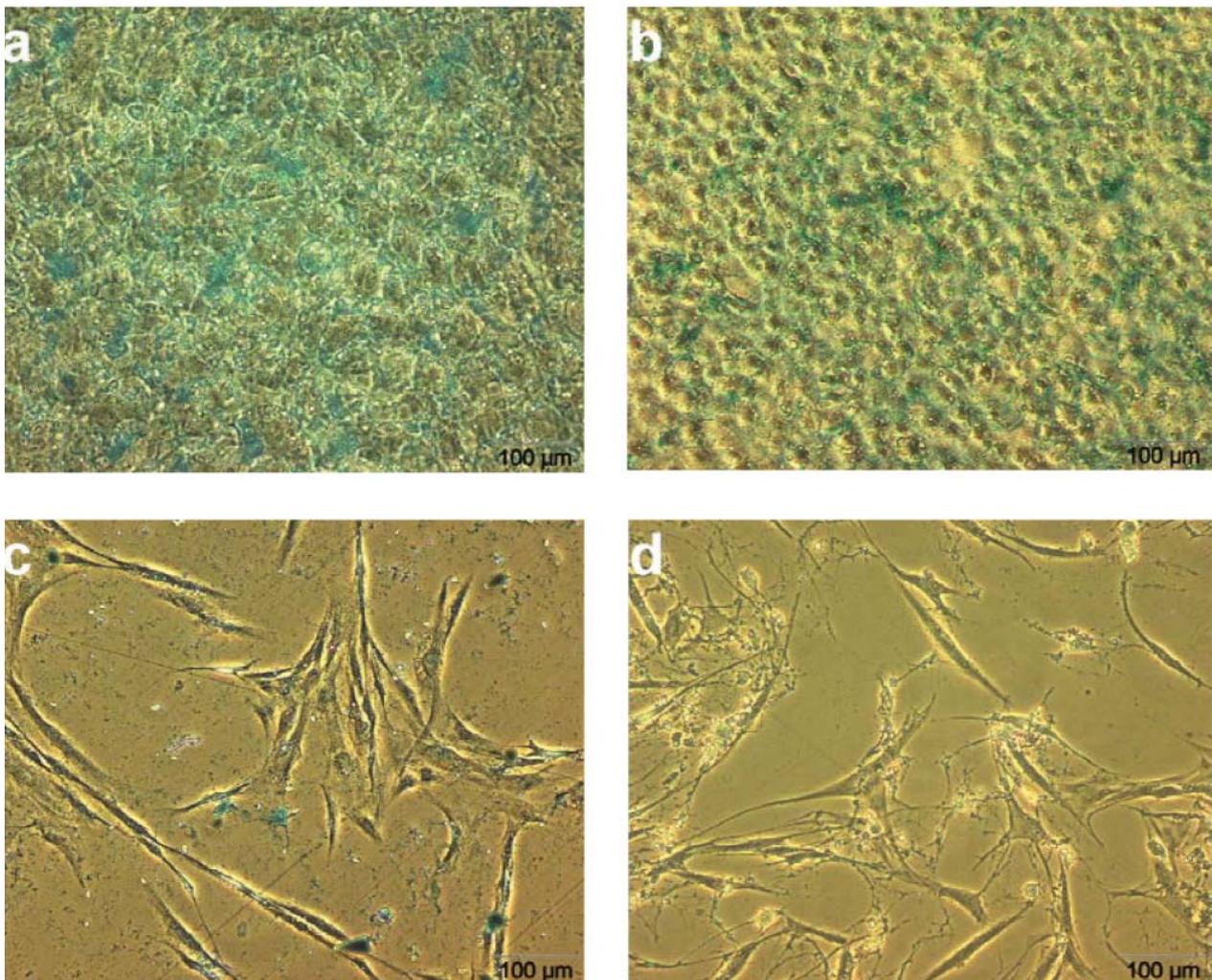
Fig. 14. Alcian blue staining at 14 d of chondrocytes **(a,b)** and of mesenchymal stem cells cultured and in the presence of gels of Pluronic F127 solely **(c)** and with rhBMP-2 **(d)**.

$F_{8,9d.f.}$= 15.36; α<0.001) confirmed that Tetronic 908, 1107 and 1307 were different from the other systems. Such an intrinsic osteoinductive effect of poloxamines was not observed for poloxamer Pluronic F127 (ALP levels superimposable with those of the negative control), which is in agreement with previous reports (Clokie and Urist, 2000; Issa *et al.*, 2008). ALP activity was also evaluated through histochemical staining as described below.

Cell differentiation and mineralization

Differentiation of mesenchymal cells to osteoblasts was visualized after ALP staining, using an inverted microscope (Fig. 7). In the negative control (no osteoinductive substance) the mesenchymal cells formed homogeneous monolayers, with a high proliferation index (Fig. 8). In the osteoinductive medium (positive control), the cells initially showed the typical spindle-shaped morphology of fibroblasts, but then the cell density diminished and the polygonal shape of osteoblasts was evident since day 7. Similarly, poloxamine gels loaded with rhBMP-2 caused differentiation to osteoblasts, which was evident at day 7 for Tetronic 908 formulations and at day 14 for the other poloxamine formulations (proliferation levels in between those of the negative and positive control, Fig. 8). On the other hand, poloxamine solely gels led

to an initial proliferation of the mesenchymal cells (first week), followed by differentiation to osteoblasts (second to third week).

Alizarin red staining at days 14 and 23 is shown in Fig. 9. Cells cultured with rhBMP-2 loaded gels evidenced the staining typical of matrix mineralization at day 14. Those treated with poloxamine solely gels did not show staining at day 14 but at day 23. No alizarin red staining occurred with Pluronic F127 solely gel. However, this poloxamer gel was highly adipogenic as observed after Oil Red O staining (Figs. 10 and 11). Cells treated with Tetronic 1307 gels solely or loaded with rhBMP-2 also evidenced a large number of lipid vesicles. Fewer vesicles were seen for Tetronic 1107 gels without rhBMP-2. No staining was observed with Tetronic 908, neither solely nor with rhBMP-2.

Alcian blue staining was negative for all cell cultures and formulations and no evidence of the typical extracellular matrix of chondrocytes with sulfated glycosaminoglycan was found (Figs. 12 and 13). Some minor blue points observed in photos of cells cultured in the presence of the poloxamines are artifacts caused by some residues of the staining. Furthermore, inverted optical microscopy observation of the cells did not reveal morphological changes of the mesenchymal cells to
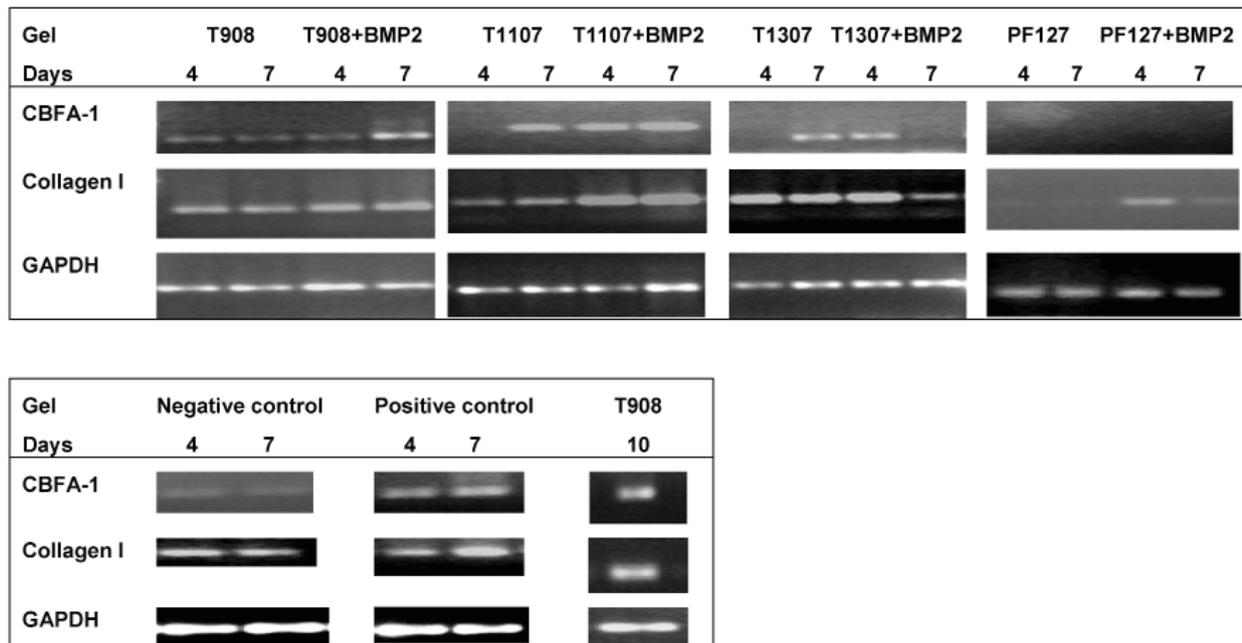
Fig. 15. Increase in osteogenic marker genes expression induced by poloxamines Tetronic 908, 1107 and 1307 and poloxamer Pluronic F127, both in the absence and in the presence of rhBMP-2. Osteogenic marker genes expression was observed using RT-PCR at various days. The expression of GAPDH was used to normalize gene expression levels.

chondrocytes (i.e., polyhedral shape; Moses *et al.*, 1992) (Fig. 14 a,b). Pluronic F127 did not induce differentiation of mesenchymal stem cells to chondrocytes either (Fig. 14 c,d).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis
Mesenchymal stem cells in culture medium (negative control) exhibited a basal expression of CBFA-1. Cells treated with Tetronic 908, 1107 and 1307 gels with and without rhBMP-2 increased mRNA expression of CBFA-1 in a time-dependent manner (Fig. 15). In the absence of rhBMP-2, the expression induced by Tetronic 1107 and Tetronic 1307 was evident at day 7, while with Tetronic 908 the induction was clearly observed latter, at day 10. In the presence of rhBMP-2, the mRNA expression of CBFA-1 occurred at day 4 with Tetronic 1107 and 1307 and at day 7 with Tetronic 908 formulations.

Negative controls showed a moderate expression of the collagen type I marker, but a significant inductive effect was recorded for Tetronic 908 with and without rhBMP-2 (particularly relevant at day 10), and for Tetronic 1107 with rhBMP-2 and Tetronic 1307 both with and without rhBMP-2 at days 4 and 7. Correspondingly, cells cultured with Pluronic F127 did not show induction of these osteogenic marker genes at any time point of the study.

Discussion

Cytocompatibility screening
Poloxamines have received less attention than their linear counterpart poloxamers in the pharmaceutical and biomedical fields in general, but particularly as components

of scaffolds or drug depots (Sosnik et al., 2008; Alvarez-Lorenzo et al., 2010b). Thus, the first step of the work was to gain an insight into the cytocompatibility of poloxamines and the incidence of their architecture variables (such as EO/PO ratio and molecular weight) and to compare the data with those obtained with the related poloxamers. Namely eight varieties of Tetronic were chosen to cover from 1,650 to 25,000 Da molecular weight range and from 0.15 to 5.27 EO/PO units ratio (Table 1). For comparison purposes we have chosen Pluronic F127, which has been already used as inactive ingredient of approved drug products and possess a molecular weight similar to that of Tetronic 1107 and relative weight content in EO of around 70% as Tetronic 1107 and 1307 (Table 1).

Balb/3T3 fibroblasts and CHO-K1 epithelial-like cells are widely used for the testing of new biomaterials, but fibroblasts are more demanding regarding nutritional conditions and more sensitive to toxic agents (Park *et al.*, 2002). Poloxamine concentrations were chosen to cover a wide range of values below and above the critical micellar concentrations (CMC), which are around 0.7% for Tetronic 901, 904 and 150R1, 1% for Tetronic 1107, 1301 and 1307, and 2% for Tetronic 304 and 908 (Gonzalez-Lopez *et al.*, 2008). No clear dependence of cell viability on CMC values was noticed. As a general trend, the hydrophilic poloxamines were more cytocompatible than the hydrophobic ones, showing better cytocompatibility as their molecular weight increases (Fig. 2). The lower cytocompatibility observed for Tetronic 901 and 904 can be related to their relatively low molecular weight and high hydrophobicity, which may enable the copolymer chains to intercalate in the cell membranes causing some disruption, as reported for Pluronic L61 of 2000 Da and HLB 1-7 (Exner *et al.*, 2005). The high cytotoxicity

recorded for Tetronic 904 at 72 h is in agreement with our previous observation of the deleterious effect of Tetronic 904 on Caco-2 monolayers integrity (Alvarez-Lorenzo *et al.*, 2010a). Cytotoxicity is commonly evaluated using the 0, 1, 2, 3 and 4 scale, which corresponds to >90%, 70-90%, 40-70%, 10-40%, and <10% cell counts compared to control growth (Xian, 2009). The positive control had a cytotoxicity scale of 4. An acceptable level of viability is of 0 or 1 in the scale, and most poloxamines fulfilled that score at 24 h except Tetronic 304 and 904 at the highest concentrations evaluated. After 72 h, Tetronic 901 and 904 had scores of 2 and 4, respectively, against CHO-K1 cells. In the case of Balb/3T3 the score of 0 or 1 was maintained by Tetronic 908 at any concentration and by Tetronic 304, 1107, 1301, 1307 and 150R1 except at the highest concentrations tested. Taking into account the cytocompatibility results, Tetronic 908, 1107 and 1307 were chosen as representatives of hydrophilic poloxamines and Tetronic 1301 as representative of the hydrophobic varieties for further studies.

Pluronic F127 showed high cell viability at 24 h, disregarding the copolymer concentration, but at 72 h a marked dose-dependence was observed, leading to values similar to those of Tetronic 1107 against Balb/3T3 fibroblasts and those of Tetronic 901 against CHO-K1 cells. It has been previously reported that poloxamer varieties show certain cell toxicity, which is particularly evident when the concentration surpasses the CMC. This effect has been related to the increase in the fluidity of the cell membrane, the inhibition of efflux pumps and cell detoxification mechanisms, and the depletion of intracellular ATP (Exner *et al.*, 2005; Krupka *et al.*, 2009).

Experiments with SAOS-2 cells (a human osteoblast cell line) revealed that Tetronic 908, 1107, 1301 and 1307 at the concentration required for the *in situ* gelling (20% w/w) lead to viability above 90% (Fig. 3). Although the four poloxamines could not be tested under exactly the same experimental conditions and the direct comparison of the cytotoxicity data may be not rigorous, the results obtained prove that these poloxamines are highly cytocompatible. Thus, Tetronic 908, 1107, 1301 and 1307 may be suitable for developing injectable scaffolds.

Temperature-induced gelling and BSA control release
Below 20 ºC, 20% w/w poloxamine solutions behaved as free flowing liquids and were easily syringeable owing to their low viscosity. An increase in temperature led to an increase in the viscosity of the systems and for them to undergo a sol-to-gel transition, driven by hydrophobic interactions among PPO blocks and packing of the micellar aggregates (Fig. 4 a,b). The greater gel temperature of Tetronic 908 (33 ºC), compared to Tetronic 1107, 1301 and 1307 (25 ºC), is due to its longer PEO blocks, which enhance the hydrophilicity of the copolymer. At 37 ºC, all systems behave as gels with storage modulus values (10^3-10^5 Pa) similar to those we obtained for Pluronic F127 and to those previously reported for other *in situ* gelling systems and syringeable depots for drug delivery (Chaibundit *et al.*, 2007; Jones *et al.*, 2009). The storage modulus values are also in the range of those found for preformed bone

scaffolds such as hydroxyapatite–atelocollagen composites (Pelin *et al.*, 2009). Poloxamine dispersions prepared in culture medium (D-MEM) behaved similarly to those prepared in phosphate buffer pH 7.4, although the gel temperature was slightly greater in D-MEM (Fig. 4b), due to the different nature of the salts present in both media. Importantly, autoclaving did not modify the rheological behavior of 20% w/w Tetronic solutions, which indicates that this method is suitable for sterilizing the injectable scaffolds. In general, the information available about the incidence of thermal treatments on the viscoelasticity of PEO-PPO block copolymer solutions is scarce, but it has been reported that Pluronic F127 stands quite well autoclaving (Woerle *et al.*, 2006). Nevertheless, even if the terminal sterilization by autoclaving would not be adequate for the Tetronic systems (e.g., owing to presence of a labile active substance, such as a peptide or protein), their low viscosity at 4 ºC makes sterilization by filtration possible.

Once 20% poloxamine systems were shown to be able to undergo sol-to-gel transitions at temperature below but near to 37 ºC, their ability to sustain the delivery of BSA was tested. BSA is commonly used as model protein, owing to its availability and recognized high stability in aqueous media (Hedoux *et al.*, 2009). The release test was performed in such a way that the gel was already formed when it entered into contact with the release medium, and the surface of contact (143.1 mm^2) was initially the same for all formulations. Release profiles showed a burst (first 5 h) followed by a gradual release for one week. The burst was especially sharp in the case of the most hydrophilic poloxamines (Fig. 5a$_1$). The slower delivery of BSA from Tetronic 1301 and 1307 systems may be related to their lower gel temperature, which makes the gel withstand better the progressive dilution by the release medium. An increase in poloxamine concentration did not lead to a significant decrease in the burst effect. For any formulation, the gels mixed completely with the release medium after 3 d, which indicates that under the test conditions the mean erosion/dissolution rate of the formulations is around 3.2×10^{-4} g/min/cm^2. This value is lower than that reported for Pluronic gels tested using greater interfacial area and under stirring conditions, namely 0.016 g/min/cm^2 (Anderson *et al.*, 2001).

rhBMP-2 release, bioactivity and gene expression
Optimal therapeutic dose of rhBMP-2 in human is difficult to establish because of the remarkable species-specific dose response, which makes results with animal models difficult to extrapolate to humans (Luginbuehl *et al.*, 2004), and because of the up-regulation of BMP inhibitors that may interfere in the healing mechanisms (Westerhuis *et al.*, 2005; Bessa *et al.*, 2008). The rhBMP-2 concentration chosen (0.037 mg/mL), which is in between the nanogram/mL range of the physiological growth factors and the milligram/mL range of current human therapeutic formulations (Luginbuehl *et al.*, 2004), has been shown to be suitable for *in vitro* and *in vivo* activity (Wang *et al.*, 1990; Kim and Valentini, 2002). It has been previously shown that BMP-2 is highly unstable in phosphate buffer, needing the addition of protease inhibitor and germicide

substances to prevent a fast degradation (Hsieh *et al.*, 2006). Therefore, the release medium was supplemented with sodium azide (0.02% w/v). In parallel to the *in vitro* release tests, bioactivity and gene expression assays were carried out. We observed that Tetronic 1301 gels underwent a remarkable phase separation from the culture medium during the bioactivity assay and, consequently, this hydrophobic poloxamine was discarded for further studies.

Despite that rhBMP-2 (18-30 kDa) is a protein smaller than BSA (67 kDa), rhBMP-2 release profiles resembled to some extent those of BSA (Fig. 5). Thus, the slow erosion/dissolution of the gels makes the control of rhBMP-2 release possible, being poloxamine gels as efficient as, or even more than, non-syringeable complex matrices reported as BMP-2 eluting scaffolds (Rai *et al.*, 2005; Hsieh *et al.*, 2006). Previous studies have shown that efficient scaffolds should combine a reasonably high initial release with a sustained delivery behavior latter on (Uludag *et al.*, 1999). Although the current state of the art indicates that there is not one single pharmacokinetic profile predictive of success, it is apparent that the extremes of release rates (bolus injections or prolonged low level release) are not beneficial to bone induction (Geiger *et al.*, 2003). rhBMP-2 release rate *in vivo* from poloxamine systems is expected to occur at slower rates than *in vitro*, because the smaller volume of medium and the quasi-static conditions. Nevertheless, the sustained delivery for almost two weeks observed *in vitro* (Fig. 5b) resembles to some extent to the *in vivo* profile reported for the commercially available collagen sponge-based rhBMP-2 formulations (Li and Wozney, 2001). Due to the temperature-induced gelling, poloxamine formulations are suitable for preparing injectable formats that enable minimally invasive application without requiring an open procedure, as most biodegradable and non-biodegradable matrices demand. Furthermore, formulation of rhBMP-2 in poloxamine systems under mild conditions has the advantage of avoiding the loss of biological activity of the growth factor, which usually happens during formulation processes that involve harsh conditions, such as heating, sonication and organic solutions. Bioactivity assays were also carried out to gain insight into this aspect.

Mesenchymal stem cells cultured in non-osteogenic medium (negative control) showed certain ALP activity, with a maximum at day 7, as previously reported (Harris *et al.*, 2004). Cells cultured in medium with Pluronic F127 solely, Tetronic 1107 solely and Tetronic 1307 with rhBMP-2 showed at day 7 an ALP activity not statistically different from that of the negative control. By contrast, ALP activity in the presence of Tetronic 908 and 1107 gels loaded with rhBMP-2 resembled that achieved with the osteogenic medium, with a maximum at day 7 (Kim and Valentini, 2002). Tetronic 1307 gel loaded with rhBMP-2 caused an increase in ALP activity since day 3, which remained practically constant for two weeks (Fig. 6). The differences in ALP activity recorded for the gels loaded with rhBMP-2 are related to the different ability of poloxamines to regulate the release of rhBMP-2 in its bioactive form. Compared to Tetronic 908 and 1107 formulations, Tetronic 1307 gel showed the most sustained delivery of protein. This slower release may lead to, given

a longer time, protein in the culture medium available for inducing cell differentiation, resulting in a prolonged plateau in ALP activity (Fig. 6).

The osteoinductive capability of the three poloxamines on their own was clearly seen when the cells were cultured in the presence of gels that did not contain rhBMP-2 (Fig. 6). For example, Tetronic 908 solely gels rendered ALP activity at day 7 as high as the osteogenic medium. This behavior contrasts with that of Pluronic F127, which did not cause any increase in the ALP activity of the mesenchymal stem cells. The remarkable ALP activity of poloxamine alone solutions can be related to its structural similarities with certain natural polyamines, such as spermine and spermidine. Natural polyamines are essential for a number of functions in the cell, including proliferation and apoptosis; however, their explicit role in these cellular processes is mostly unknown (Helder and Wuisman, 2007). Particularly, polyamines at concentrations in the range of 1 nM to 100 μM have been recently shown to induce proliferation and differentiation of stem cells (Helder and Wuisman, 2007). Furthermore, since it is well known that the alkaline phosphatases are most effective in an alkaline environment (Wahlstroem *et al.*, 2007), the base-like character of polyamines and poloxamines may assist their osteoinductive performance. The osteogenic efficiency of poloxamines by themselves was confirmed in further studies described below. The slower bioactivity of poloxamine solely gels (compared to those loaded with rhBMP-2) correlates with the fact that the copolymer depot erodes/dissolves in the cell culture medium more slowly than rhBMP-2 is released. Once differentiation occurs, the ALP activity returns to basal levels. This explains why various maxima in the ALP activity were not observed for rhBMP2-loaded poloxamine gels. The ALP activity induced by poloxamine gels both with and without rhBMP-2 was clearly visualized by cell histochemical staining at day 14 (Fig. 7). Such an osteogenic activity of poloxamines may offer novel perspectives in the field of the fracture healing, overcoming the safety and the cost/effectiveness concerns associated to the large scale clinical use of recombinant growth factors.

In contrast to that observed in the absence of osteoinductive agents, mesenchymal stem cells in the osteoinductive medium (positive control) did not proliferate but differentiated to osteoblasts (Fig. 8). This finding is in agreement with the typical increase in cell apoptosis and decrease in cell proliferative activity observed during osteogenic induction *in vitro*, due to the biochemical stress underwent by the cells in the osteoinductive medium (Niemeyer *et al.*, 2006; Li *et al.*, 2009). Poloxamine gels loaded with rhBMP-2 showed an intermediate behavior, combining proliferation and differentiation to osteoblasts. The earlier differentiation induced by Tetronic 908 formulations (evident since day 7) compared to the other formulations (seen at day 14) can be explained by the greater ALP activity at day 7 of cells cultured with Tetronic 908 gels loaded with rhBMP-2 (Fig. 6). Interestingly, poloxamines alone (without rhBMP-2) caused proliferation of the mesenchymal cells for the first week, and then differentiation to osteoblasts for the second to the third week. It should be noticed that Tetronic 908

rendered the gels with the highest proliferation level and osteoinductive capacity.

The poloxamine gels loaded with rhBMP-2 induced matrix mineralization already at day 14 (Fig. 9). This finding confirmed that the cells had previously differentiated to osteoblasts, which are able to start the formation of bone matrix and to regulate the deposition of salts. Mineralized nodules were also clearly seen in the cultures with poloxamine solely gels, although at latter time (23 d) due to the on-going differentiation of the cells to osteoblasts (Jaiswal *et al.*, 1997). By contrast, Pluronic F127 solely gels were not able to induce matrix mineralization.

Since mesenchymal stem cells can give rise to the progenitors of various cell lineages (Yin *et al.*, 2010), differentiation into adipocytes and chondrocytes was also assessed using Oil Red O and Alcian blue staining, respectively. Several previous studies have shown that rhBMP-2 can induce differentiation of mesenchymal stem cells to adipocytes (Ji *et al.*, 2000; Date *et al.*, 2004; Kato *et al.*, 2009). Furthermore, it has been reported that certain poloxamer varieties promote adipogenic differentiation of mesenchymal stem cells from bone marrow (Vashi *et al.*, 2008). To gain an insight into these aspects, we tested Pluronic F127 under the same conditions as poloxamines. This poloxamer was highly adipogenic, as was also the case for Tetronic 1307 systems, without or with rhBMP-2 (Figs. 10 and 11). This finding is in agreement with the smallest alkaline phosphatase activity recorded with Tetronic 1307 formulations. Some lipid vesicles stained with Oil Red O were also observed in the presence of Tetronic 1107 gels without rhBMP-2, but to a much lower extent than in the case of Tetronic 1307. None of the Tetronic 908 formulations were adipogenic. Although, according to the analysis on the supplier's data sheet Tetronic 908 is the largest poloxamine with an average molecular weight of 25,000 (see Table 1), it has been reported that different batches of commercial copolymers may differ remarkably from each other in mean molecular weight and polydispersity (Moghimi *et al.*, 2001; Al-Hanbali *et al.*, 2007). Analytical method and conditions may also affect to the results. This is particularly so for GPC analysis of the poloxamine batches used in the present study and under the conditions reported by Chiappetta *et al.* (2010) rendered average-number molecular weights of 9,600, 11,600 and 11,700 for Tetronic 908, 1107 and 1307, respectively. According to these experimental results, Tetronic 1307 appears to be the largest poloxamine and, consequently, the contribution of the diamine group to its osteoinductive activity may be shielded at a greater extent by the long EO-PO copolymer arms than in the case of the other poloxamines. Alcian blue staining tests revealed that neither poloxamines nor Pluronic F127 induced differentiation of mesenchymal stem cells to chondrocytes (Fig. 14).

Finally, osteogenic marker gene expression was analyzed to confirm the osteoblastogenesis induction (Trentz *et al.*, 2010). CBFA-1 (Runx-2), an osteoblast specific transcription factor, essential for developing a mature osteoblast phenotype, has been identified as the only transcriptional factor responsible for osteoblastic differentiation and expression of osteocalcin and osteopontin (Hing *et al.*, 2004). In agreement with data shown in Fig. 6 and also with previous reports (Zuk *et al.*, 2002), mesenchymal stem cells exhibited a basal expression of CBFA-1. Tetronic 908, 1107 and 1307 gels with and without rhBMP-2 increased mRNA expression of CBFA-1, although earlier in the presence of rhBMP-2 (Fig. 15). Both with and without rhBMP-2, the induction was observed later for Tetronic 908 than for Tetronic 1107 and 1307, which is in agreement with the high cell proliferation caused by Tetronic 908.

It is well-known that initial collagen matrix accumulation is essential for sequential expression of the differentiation related proteins, e.g. alkaline phosphatase, bone sialoprotein, and osteocalcin (Hutmacher and Sugunan, 2004). Again, all gels induced the expression of collagen type I marker, but Tetronic 908 ones with and without rhBMP-2 did it later. Induction was not observed with Pluronic F127. This finding may be related to the capability of the linear poloxamer to induce adipogenic differentiation, as observed with the bioactivity and histochemical analysis described above. The central ethylenediamine group in the X-shaped structure of poloxamines seems to be crucial for the osteoinductive capability of Tetronics. No other polymer has been previously reported to be osteoinductive by itself.

Conclusions

Various poloxamine varieties (Tetronic 908, 1107, 1301 and 1307) combine good cytocompatibility with the ability to undergo sol to gel transitions at near to body temperature. Moist heat sterilization (autoclaving) does not alter the rheological properties of the copolymer solutions, which is an important aspect for the development of injectable scaffolds. *In situ* formed poloxamine gels can sustain the delivery of proteins at 37 ºC (BSA and rhBMP-2) for several days; the release rate being tuneable by the hydrophilicity and the molecular weight of the copolymer. The initially rapid release of an important amount of rhBMP-2 followed by a slower release enables the gels to exhibit a marked ALP activity at day 7 and the matrix mineralization at day 14. As a remarkable novel finding, poloxamines themselves induced the proliferation of mesenchymal cells and then their differentiation to osteoblasts, although more slowly than when combined with rhBMP-2. The peculiar X-shape structure of the copolymers with a central ethylenediamine group may create an adequate environment for the ALP activity. Poloxamines are pointed out as a very promising material for syringeable *in situ* gelling systems with osteogenic performance, acting themselves as the active agent or as suitable BMP carriers. Although poloxamines are not biodegradable, it is expected that as the scaffold erodes and the copolymer reaches blood circulation, it could be rapidly excreted in the urine without toxic effects, as occurs for poloxamers. Nevertheless, *in vivo* studies are necessary to further clarify the clinical applicability of poloxamine gels.

Acknowledgements

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Discussion with Reviewer

Reviewer II: In the release studies the gel was formed at 37 ºC in test tubes and the phosphate buffer solution was added over the mass formed. Please include more details on the geometry of the gel samples and on the surface available between the gel samples and the liquid medium, as these aspects can influence the release profile.

Authors: Since the poloxamine solutions underwent the sol to gel transition inside the test tubes, the gels adopted a cylindrical shape having a diameter equals to the inner diameter of the test tube. The inner diameters of the test tubes and the surface area of the gel in contact with the release medium were 13.5 mm and 143.1 mm^2 respectively.

Reviewer II: The authors present rheological data performed on well established systems. How they can be compared with other rheological results previously reported in the literature?

Authors: At 37 ºC, Tetronic 908, 1107, 1301 and 1307 systems behave as gels with storage modulus ranging between 10^3 and 10^5 Pa. These values, which are similar to those we obtained for Pluronic F127, are in the range of those previously reported for other *in situ* gelling systems and syringeable depots for drug delivery (Chaibundit *et*

al., 2007; Jones *et al.*, 2009, text references). Furthermore, the storage modulus values are in the range of those found for preformed bone scaffolds such as hydroxyapatite–atelocollagen composites (Pelin *et al.*, 2009, text reference).

Reviewer II: The release profile of both BSA and BMP-2 are given in cumulative (total) mass released. What is the correspondence of such values with respect to the total amount of proteins initially present in the gel samples? Could the authors indicate for both BMP-2 and BSA what % was released after 1 d and at the last time point? Could the authors comment on this release profiles? Are the release profiles adequate in respect of the application? How do these systems compare to the ones available in the literature?

Authors: 100% BSA and BMP-2 released correspond to 10 and 0.06 mg, respectively. Regarding BSA, Tetronic 908 and 1107 formulations released 50-60% at 24 h and nearly 90% at day 7, while Tetronic 1301 and 1307 formulations released 30-35% at 24 h and ca. 75% at day 7. Regarding BMP-2, Tetronic 908, 1107 and 1307 gels released 58, 41 and 32% at 24 h and 75, 55 and 82% at day 7, respectively. Although a pharmacokinetic profile predictive of success has not been identified yet, previous studies have shown

that efficient scaffolds should combine a reasonably high initial release with a sustained delivery behavior later on (Uludag *et al.*, 1999, text reference). Therefore, the poloxamine-based formulations seem to be suitable for the delivery of rhBMP-2.

Reviewer II: The authors should comment on the applicability of such materials as matrices for the delivery of growth factors taking into account that they are not biodegradable.

Authors: It has been previously reported that poloxamers injected intravenously are rapidly excreted in the urine, with minor accumulation in lung, liver, brain, and kidney tissue, and no risk of reproductive and/or developmental toxicity has been found (Cosmetic Ingredient Review (CIR) Expert Panel, 2008, text reference). Since poloxamines possess a polyoxyethlyene-polyoxypropylene block composition similar to that of poloxamers, non-biodegradable poloxamine matrices are expected to erode releasing the copolymer to the blood circulation, from which the poloxamines could be rapidly cleared through kidney filtration.