The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma
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Abstract
Human hepatocellular carcinoma (HCC) is one of the major causes of death worldwide. Targeted uptake of therapeutic agent in the cell-, tissue- or disease-specific manner represents a potential technology for the treatment of HCC. A new docetaxel-loaded hepatoma-targeted solid lipid nanoparticle (tSLN) was designed and prepared with galactosylated dioleoylphosphatidyl ethanolamine. The cellular cytotoxicity, cellular uptake, subcellular localization, in vivo toxicity, therapeutic effect, biodistribution and histology of tSLNs were investigated. The tSLNs showed the particle size about 120 nm with encapsulation efficiency >90%, a low burst effect within the first day and a sustained release for the next 29 days in vitro. Cytotoxicity of tSLNs against hepatocellular carcinoma cell line BEL7402 was superior to Taxotere® and non-targeted SLNs (nSLNs). The tSLNs also showed better toleration and antitumor efficacy in murine model bearing hepatoma compared with Taxotere® or nSLNs. The studies on cellular uptake and biodistribution indicated that the better antitumor efficacy of tSLNs was attributed to both the increased accumulation of drug in tumor and more cellular uptake by hepatoma cells. The histology demonstrated that tSLNs had no detrimental effect on both healthy liver and liver with fibrosis. These results implied that this targeted nanocarrier of docetaxel could enhance its antitumor effect in vivo with low systemic toxicity for the treatment of locally advanced and metastatic HCC.

1. Introduction
Primary liver cancer is a major health problem worldwide. Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and the third most common cause of cancer-related death [1]. Treatments of HCC are conventionally divided into curative and palliative. Surgical resection is the major curative technique, but it is very limited for patients with multiple or metastatic tumors [2]. Therefore, it is of great importance to search for effective chemotherapeutic agents to improve the survival rate of patients with advanced or recurrent HCC after surgical treatment.

Docetaxel is a new taxoid, structurally similar to paclitaxel, but more effective as inhibitor of microtubule depolymerization [3]. Docetaxel combined with some chemotherapeutic drugs showed high anticancer efficacy in patients with breast, pancreatic, gastric and urothelial carcinomas [4–7]. Although docetaxel produced profound effects on several hepatocellular carcinoma cells in vitro, the clinical test didn't show satisfactory effect in patients with advanced HCC and liver cirrhosis, mainly due to that docetaxel lacks tissue and cell specificity [8–10]. As a result, a drug delivery system targeted to the liver, especially hepatocellular carcinoma cells, represents an attractive prospect for future therapy.

Nanoparticles can be delivered to specific sites by size-dependent passive targeting [11]. It has been reported that nanoparticles injected intravenously are taken up by the liver after only a few minutes due to the opsonization process [12]. To obtain higher selectivity and enhance the uptake of nanoparticles into the target cells, active targeting could be an effective approach and could be achieved by modifying the nanoparticles with a ligand that can bind to the asialoglycoprotein (ASGP) receptor, which is highly expressed on the surface of several human hepatoma cell lines. Galactosylation on particle surface can facilitate binding of particle with ASGP receptors and subsequent uptake into hepatoma cells [13–15].

We postulated that docetaxel-loaded nanoparticles targeting to hepatoma cells could bring about enhanced cytotoxicity and anti-tumor efficacy with better tolerance in vivo for treatment of HCC. To validate the hypothesis, in this work, we designed docetaxel-loaded solid lipid nanoparticles (SLNs), which mainly consisted of biodegradable and biocompatible components-egg yolk
phosphatidylcholine (ePC) and dioleoylphosphatidyl ethanolamine (DOPE). For specific targeting to ASGP receptor on hepatocellular carcinoma, a galactose moiety was used by conjugating DOPE with lactobionic acid (LA). Hepatoma-targeted solid lipid nanoparticles (tSLNs) were prepared by homogenization at elevated temperature, and the physicochemical characteristics of tSLNs were determined. In vitro cytotoxicity of tSLNs was examined by MTT assay on hepatoma cell line BEL7402. Cellular uptake and intracellular distribution studies were performed using rhodamine B as a model hydrophobic fluorescent probe. The maximum tolerated dose (MTD), antitumor efficacy and biodistribution were investigated on nude mice bearing hepatoma. Since tSLNs were supposed to accumulate mainly in liver, the risks of liver damage were also evaluated on both healthy mice and mice with liver fibrosis and cirrhosis.

2. Materials and methods

2.1. Materials

Docetaxel was obtained from Sanwei Pharmaceutical Co. Ltd. (Shanghai, China). Egg yolk phosphatidylcholine (ePC), dioleoylphosphatidyl ethanolamine (DOPE), trimyristin (TM), lactobionic acid (LA) and the other reagents (analytical grade) were purchased from Sigma (St. Louis, MO, USA).

BEL7402 hepatocellular carcinoma cell line was obtained from Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China) and grown in RPMI 1640 medium (Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS). C57BL6 mice and female nude mice were purchased from Shanghai Experimental Animal Center (Shanghai, China). All animal procedures were performed following the protocol approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Galactosylated DOPE (Gal-DOPE) was synthesized by coupling lactobionic acid with the amino terminal of DOPE [16]. Briefly, 126 mg (0.17 mmol) DOPE and 65 mg (0.17 mmol) lactobionic acid in 6 ml of MeOH were refluxed for 24 h. The product was purified by chromatography and confirmed by 

2.2. Preparation and physicochemical characteristics of tSLNs

SLNs were prepared by homogenization method at elevated temperature (65°C [17]). Briefly, TM, ePC, Gal-DOPE and docetaxel (120 mg) were added at a ratio of 90:30:3:6 (w/w) for tSLNs or 90:30:0:6 (w/w) for non-targeted solid lipid nanoparticles (nSLNs). The nanoparticles were purified by a 3 x 15 cm alumina column and lyophilized.

Size, size distribution and ζ potential of SLNs were measured by laser light scattering using a Nippon 380/SLS zeta potential analyzer (Particle Sizing System, USA) after SLNs were dispersed in water.

The entrapment efficiency was obtained by measuring the amount of docetaxel that was encapsulated in SLNs. Briefly, 10 mg of SLNs was dissolved in 2 ml of methanol and determined by HPLC method following experimental conditions: Diamond C18 column (150 mm × 4.6 mm i.d., pore size 5 μm), the mobile phase: CH3CN:H2O (1:1, v/v), flow rate: 1.0 ml/min, and measured wavelength: 230 nm.

The dialysis method was applied to monitor the release of docetaxel from SLNs at the presence of bovine serum albumin (BSA) as described previously [17–19]. SLNs (10 mg) were suspended in 1.5 ml PBS (pH 7.4) in a dialysis bag (molecular cutoff of 5000 Da) and were incubated in 50 ml PBS (50 mg/ml BSA, pH 7.4) at 37 °C under horizontal shaking (300 rpm, Thermomixer, Eppendorf). At predetermined time intervals, the aliquots of incubation medium were removed and the same volume of fresh solution was added. The amount of docetaxel released in each time point was determined by the HPLC assay as described above.

2.3. In vitro cellular cytotoxicity assays

The cytotoxicity of SLNs was evaluated by MTT assay on BEL7402 cell. Taxotere®, tSLNs or nSLNs were added at equivalent docetaxel per well in RPMI 1640 medium. SLNs without docetaxel were added as control. Absorbance was measured at 490 nm using a microplate reader (ELX 800; BIO-TEK Instrument, Inc.). The cell viability (%) was calculated and compared with the untreated control.

2.4. Cellular uptake experiment

Rhodamine B was encapsulated in nanoparticles as a probe for the uptake study and confocal laser scanning. Free rhodamine B was removed from the rhodamine B-loaded nanoparticles via ultrafiltration. BEL7402 cells were seeded in 24-well plates and incubated for 24 h before use. Rhodamine B labeled nSLNs (RB/nSLNs) or Rhodamine B labeled tSLNs (RB/tSLNs) were added at equivalent rhodamine B per well and incubated with cells for 2 h.

To investigate the endocytotic mechanism that was responsible for internalization of SLNs, BEL7402 cells were incubated for 60 min with 10 μm nystatin (inhibitor of caveolar-mediated endocytosis), 56 μm chlorpromazine (inhibitor of clathrin-mediated endocytosis) or 3 μm cytochalasin D (inhibitor of macropinocytosis) prior to incubation with rhodamine B labeled nanoparticles. Cells were collected, washed and resuspended in PBS. The fluorescence was measured using a FACScalibur (Becton Dickinson, USA). All experiments were performed in triplicate.

2.5. Subcellular localization

BEL7402 cells were seeded on 10-mm² glass coverslips placed in 24-well plates and incubated for 24 h. RB/nSLNs and RB/tSLNs were added at equivalent rhodamine B per well and incubated with cells for 2 h. The cells were washed twice with PBS, stained by Lysotracker Green DND-26 (from Molecular Probes, Eugene, OR) for 5 min and Hoechst 33342 for 30 min, and fixed with 4% paraformaldehyde immediately. Subcellular localization was determined using confocal microscopy (Leica Microsystems Inc.).

2.6. In vivo experiments

2.6.1. Toxicity of SLNs

MTD of Taxotere® and docetaxel-loaded SLNs administrated intravenously was determined in healthy female nude mice. Survey experiments to define the MTD were performed with two animals per group. Doses were escalated in twofold increments starting at 10 mg/kg. Drug effects were determined by close observation of weight changes and survival. The highest non-lethal dose of docetaxel causing <10% weight loss within 1 week of cessation of dosing was defined as the MTD. Animals showing weight loss exceeding 20% were sacrificed, as changes of this magnitude often indicate lethal toxicity. After the survey experiment, the approximate MTD was refined further using three groups (n = 10).

2.6.2. Therapeutic experiments

Subcutaneous tumors on the left flank of female nude mice (n = 50) were initiated by injection of 1 x 10⁶ viable cells in a volume of 0.1 ml. Tumors were allowed to grow for approximately 5 days to a volume of 100–200 mm³. Tumor-bearing mice were randomly assigned to one of the following groups (n = 5–6): saline, Taxotere®, nSLNs or tSLNs. Animal weight and tumor volume were measured five times weekly until the tumor volume reached 5000 mm³, then the animals were sacrificed for humane reasons. Tumor volume was determined by measuring three orthogonal diameters of the tumor and was calculated as one-half of the product of the diameters.

2.6.3. Biodistribution of SLNs

Female nude mice (n = 30) bearing hepatoma (100–200 mm³) were randomly assigned to three groups (n = 10) and injected intravenously through the tail vein with tSLNs (10 mg/kg), nSLNs (10 mg/kg) or Taxotere® (10 mg/kg). In each group, mice were sacrificed at 0.5 or 6 h after drug administration (n = 5 at each time point). Tumor, liver, kidney, lung, heart and spleen were collected. All the tissues were homogenized and docetaxel was extracted with methanol. The amount of docetaxel in each tissue was determined by the HPLC assay as described above. The data were normalized to the tissue weight.

2.6.4. Histology

C57BL6 mice were randomly divided into two groups. One group was used for liver fibrosis model, which was induced by injecting CCl₄ (1 ml/kg; 1:1 in mineral oil) subcutaneously twice weekly for 4 weeks [20]. All the mice then received tSLNs at a dose of 20 mg/kg through the tail vein once weekly for 3 weeks. Liver samples were harvested on the day 7 of the third week and fixed with 4% periodate–lysine–paraformaldehyde (PLP) solution and then transferred successively into solutions of 10–20% sucrose in 0.1 mol/l phosphate buffer, followed by embedding in paraffin. Sections of 5-μm thickness were prepared for hematoxylin

Fig. 1. 1H NMR spectra of galactosylated dioleoylphosphatidyl ethanolamine in CD3OD.
and eosin (H and E) stains. Histological analysis was performed in a blind manner by an expert.

2.7. Statistical analysis

Statistical tests were performed with Student’s t test, the Wilcoxon signed rank test, or the Mann–Whitney test (SPSS software, version12.0, SPSS Inc.). When differences were detected, the Wilcoxon signed rank test was used to test for pairwise differences between treatment groups and the Mann–Whitney test was used to determine the differences between independent sample groups. p Values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Physicochemical characteristics of SLNs

The mean particle size of tSLNs was 120.4 ± 10.4 nm (polydispersity index 0.236 ± 0.008) with zeta potential −12.4 ± 1.1 mV, entrapment efficiency 92.5 ± 3.7% and the loading 20.9 ± 1.0% (w/w). As control, nSLNs showed the mean particle size 118.2 ± 9.6 nm (polydispersity index 0.193 ± 0.003), zeta potential −13.2 ± 1.3 mV, entrapment efficiency 94.3 ± 4.1% and the loading 21.2 ± 1.2% (w/w), which did not show significant difference compared with tSLNs (p > 0.05).

In vitro release profiles were obtained by representing the percentage of drug released with respect to the amount of docetaxel encapsulated in SLNs (Fig. 2). The tSLNs exhibited a low burst effect with about 18.2% drug released within the first day, which indicated that docetaxel incorporated in tSLNs was likely to remain association with the nanoparticles and was taken up into the cells as the particles rather than as free drug. After 1 day, docetaxel release profiles displayed a sustained release phase. The amount of cumulated drug released over 30 days was 83.4%. This sustained release could mainly result from the erosion and degradation of the components of nanoparticles. Compared with tSLNs, nSLNs exhibited higher burst effect with about 23.6% drug release within 1 day, and the amount of cumulated drug released over 30 days was 88.6%.

3.2. In vitro cytotoxicity

In order to know the activity of docetaxel-loaded nanoparticles, in vitro cellular cytotoxicity was evaluated by MTT assay. The viability of BEL7402 cells treated with tSLNs did not demonstrate a significant difference compared with Taxotere\textsuperscript{®} at low docetaxel concentrations (<10 nM, p > 0.05), but the inhibiting activity of...
tSLNs increased with docetaxel concentration from 10 to 50 nM, and demonstrated a significant difference at 50 nM compared with the activity of Taxotere® (p < 0.01) (Fig. 3). On the contrary, nSLNs displayed a significantly higher viability compared with Taxotere® at drug concentration from 1 to 50 nM (p < 0.01). In addition, blank nSLNs and tSLNs without docetaxel showed neglectable cytotoxicity.

3.3. Cellular uptake and subcellular localization

The in vitro release profiles of rhodamine-loaded SLNs or tSLNs (data not shown) showed neglectable difference from those of docetaxel-loaded SLNs or tSLNs, which indicated that rhodamine incorporated in tSLNs remained association with the nanoparticles and was taken up into the cells as particles. As shown in Fig. 4A, a little fluorescence was detected in cells incubated with RB/nSLNs, whereas much higher fluorescence intensity was shown in cells treated with RB/tSLNs, which could be because only a little RB/nSLNs was internalized into the cells due to the electrostatic repulsion between RB/nSLNs and cellular membrane with negative charge. While in the case of RB/tSLNs, internalization could be enhanced via the ligand-receptor recognition. It could be speculated that the higher cytotoxicity of tSLNs compared with nSLNs was mainly result from better internalization.

In order to know the endocytotic pathway involved in the internalization of nanoparticles, we tested whether a specific endocytotic inhibitor could block the internalization. As shown in Fig. 4B, the internalization of RB/tSLNs was obviously inhibited by chlorpromazine and partly by asialofetuin (ASF), a natural ligand of ASGP receptor, which indicated that the internalization of tSLNs was mainly mediated by ligand-receptor recognition and clathrin-mediated endocytosis. In contrast, the internalization of RB/nSLNs seemed to be mediated by all the three pathways rather than a specific one (Fig. 4B).

![Confocal images of BEL7402 cells after treatment of RB/nSLNs (A) and RB/tSLNs (B) or after the triple fluorescence-labeling experiments: red fluorescence from Rhodamine B for RB/tSLNs (C), green fluorescence from LysoTracker Green DND-26 for endosomes (D) and blue fluorescence from Hoechst 33342 for nuclear (E). Colocalization of red and green fluorescence was observed in cells (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).](image)
Confocal microscopy was used to observe the intracellular distribution of the internalized nanoparticles. After incubation with either RB/nSLNs or RB/tSLNs, the fluorescence was mainly localized in cytoplasm (Fig. 5A and B). Fluorescence intensity of cells treated with RB/tSLNs was much higher than that of cells treated with RB/nSLNs, which was consistent with above result of cellular uptake. We also performed the triple fluorescence-labeling experiments and visualized green fluorescence from Lysotracker Green DND-26 for endosomes and blue fluorescence from Hoechst 33342 for nuclear. Colocalization of red and green fluorescence was observed in cells treated with RB/tSLNs for 30 min (Fig. 5F), which demonstrated that the nanoparticles were localized in the endosomes after internalization.

3.4. MTD and in vivo therapeutic experiment

The MTD of tSLNs, nSLNs and Taxotere® administrated by intravenous route was determined in healthy nude mice prior to the therapeutic experiment. The result showed that tSLNs and nSLNs were well tolerated with no obvious side effects (quick wake-up, signs of respiratory distress). tSLNs and nSLNs did not show significant difference with the MTD about 260 mg/kg (docetaxel dose) after single dose, whereas the MTD of Taxotere® was 80 mg/kg, and Taxotere® induced severe prostration, apathy, respiratory distress and catatonia at this dose level. The blank SLNs without docetaxel didn’t show any obvious toxicity (data not shown).

Antitumor activity was evaluated at a dose of 10 mg/kg administrated by intravenous route. tSLNs, nSLNs or Taxotere® (10 mg/kg) was administrated on day 6 after tumor implantation. Fig. 6A showed that Taxotere® and SLNs could inhibit tumor growth, in particular, tSLNs showed stronger tumor regression with a significant difference compared with Taxotere® (p < 0.01) or nSLNs (p < 0.05) on day 21. Unfortunately, tumor growth could not be inhibited completely by this single dose (Fig. 6A).

In order to enhance antitumor effect further without increasing the toxicity, a schedule of multiple dosing (10 mg/kg x 3 times) was performed. Animals were dosed on day 6, 13 and 20 after tumor inoculation. The tumor size and body weight were monitored for 30 days (Fig. 6B). In saline group, the treatment did not show any long-term efficacy, and the relative tumor volume (RTV) at the end of the experiment was 23.1 ± 2.3. Taxotere® delayed the tumor growth significantly with the final RTV 7.2 ± 0.1, but the body weight decreased obviously (about 8% of original weight, p < 0.05), which indicated the severe toxicity of Taxotere®. In nSLNs-treated group, a better antitumor activity with neglectable toxicity compared with Taxotere® group was observed. The tSLNs-treated group demonstrated the most dramatic efficacy, and all of the six animals showed complete tumor regression (final RTV = 0) with no significant toxicity.

Fig. 6. Antitumor effects (in terms of tumor growth) of tSLNs, nSLNs, Taxotere® or saline on nude mice bearing hepatoma after single dose (A) or a schedule of multiple doses (B). Data were given as mean ± SD (n = 5–6). Statistical significance compared with Taxotere® group: *p < 0.05 and **p < 0.01.

Fig. 7. Tissue distribution of docetaxel in female nude mice bearing hepatoma at 0.5 h (A) and 6 h (B) after tSLNs, nSLNs and Taxotere® were injected intravenously through the vein. Data were given as mean ± SD (n = 5). Statistical significance compared with Taxotere® group: *p < 0.05 and **p < 0.01.
3.5. Biodistribution of SLNs

Biodistribution of docetaxel-loaded nanoparticles in nude mice bearing hepatoma was determined at 0.5 h or 6 h after injection. As shown in Fig. 7, drug in Taxotere® was mainly distributed to the heart at 0.5 h after injection, which might cause the acute toxicity, and then to kidney, lung, spleen and liver, respectively. The concentration of docetaxel in tumor was very low. The accumulation of nSLNs and tSLNs in liver was 2.1 and 2.7 times higher than that of Taxotere® at 0.5 h, respectively and the amount of docetaxel in heart was much lower compared with Taxotere®. The nanoparticles also showed 1.7 (nSLNs) and 2.4 (tSLNs) times higher accumulation in tumor compared with Taxotere® at 6 h after injection, respectively. These results indicated that nanoparticles could result in higher accumulation of docetaxel in liver and tumor.

3.6. Histology

In order to verify whether tSLNs impaired the liver function, both microscopic changes of hepatocytes and serum liver parameters were evaluated in mice with or without liver fibrosis. In healthy mice, tSLNs neither caused changes of hepatocytes (Fig. 8) nor modified serum liver parameters (data not shown). In mice with liver fibrosis, although a selective docetaxel accumulation in liver, the drug did not show a detrimental effect on the histology of the liver, which indicated that tSLNs would not induce a severe liver damage to patients with liver fibrosis.

4. Discussion

HCC is well known because of its resistant to chemotherapeutic drug to evade the effects of chemotherapy [21]. In addition, cancer cells in solid tumor tend to be more resistant to chemotherapy than non-aggregating cancer cells due to various permeation barriers, which make it difficult to achieve high intratumoral drug concentration in solid tumor [22]. This latter type of drug resistance may lead to the compromised clinical outcomes even though an anticancer drug shows good efficacy in vitro. As a result, to maximize the amount of drug, such as docetaxel, that can reach the hepatic tumor and subsequently be internalized into hepatoma cells would be the key point to solve this problem. The aim of this work was to develop and evaluate an alternative formulation of docetaxel for more effective therapy of HCC.

Compared with polymeric nanoparticles, solid lipid nanoparticles (SLNs) attracted more attention in recent years. SLNs could avoid the disadvantages of other colloidal carriers and exert the advantages such as to control drug release and drug targeting, to increase physical stability, high drug loading, low toxicity, avoidance of organic solvents and ease of large scale production, [23]. Therefore, the development of docetaxel-loaded SLNs should be a worthwhile and promising strategy.

The particle size shows a great effect on drug release characteristic and distribution in vivo. Nanoparticles injected by intravenous route can be taken up by the liver after a few minutes due to the opsonization process [12]. It has been reported that the solid tumor shows hypervascular permeability and impaired lymphatic drainage. So nanoparticles (<200 nm) can significantly accumulate in tumor by “filtration” mechanism [24]. Both of SLNs showed size around 120 nm and encapsulation efficiency over 90%. The size-dependent passive targeting of SLNs to liver as well as tumor was confirmed by the biodistribution study in nude mice bearing hepatoma (Fig. 7). The accumulation of tSLNs in liver and tumor was 2.7 times and 2.4 times higher than Taxotere®, respectively. Besides passive targeting, active targeting of tSLNs was designed and achieved by galactosylation on the surface of nanoparticles. The cellular uptake of RB/tSLNs was significantly higher than that of

![Fig. 8. Histological staining of liver from healthy C57BL6 mice treated with saline (A) or tSLNs (B) and from mice with fibrosis treated with saline (C) or tSLNs (D).](image-url)
RB/nSLNs and could be partly inhibited by the excess ASF, indicating that ASGP receptor-mediated endocytosis was one of the major routes of the internalization of galactosylated nanoparticles. The tSLNs showed better antitumor efficacy compared with Taxotere® on nude mice bearing hepatooma, which could be attributed to following reasons: (1) docetaxel-loaded SLNs showed much better toleration in vivo compared with Taxotere®. Efficacy and therapeutic value of antitumor drug could be substantially demonstrated that tSLNs did not bring about detrimental effect on both healthy liver and liver with fibrosis in vivo. In a word, tSLNs could be a safe and efficient chemotherapeutic agent for the treatment of locally advanced and metastatic HCC.

5. Conclusions

The tSLNs showed the particle size about 120 nm with encapsulation efficiency >90%, a low burst effect within the first day and a sustained release for the next 29 days in vivo. Cytotoxicity of tSLNs against hepatocellular carcinoma cell line BEL7402 was superior to Taxotere® and nSLNs. The tSLNs also showed better tolerant and antitumor efficacy in murine model bearing hepatooma compared with Taxotere® or nSLNs. The better antitumor efficacy of tSLNs was attributed to both the increased accumulation of drug in tumor and more cellular uptake by hepatoma cells. The histology demonstrated that tSLNs had no detrimental effect on both healthy liver and liver with fibrosis. These results implied that this targeted nanocarrier of docetaxel could enhance its antitumor effect in vivo with low systemic toxicity for the treatment of locally advanced and metastatic HCC.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 5 and 6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2008.09.014.

References