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The use of gelatin as a vehicle for drug and peptide delivery

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Gelatin, a naturally occurring polymer, has been investigated as a vehicle for drug delivery in two different delivery systems: microspheres and as a coating on titanium implants. The gelatin was loaded with recombinant human growth hormone (hGH) which was dispersed within the polymer matrix prior to crosslinking; it was then made into microspheres or coated onto the implants. The release of hGH was monitored *in vitro* using an "in-house" ELISA system. The effects of pH on the swelling kinetics and the physical properties of the loaded gelatin in the microsphere system were studied. In addition, the effect of ultrasound on the microspheres was investigated as a possible method for controlling the rate of release of hGH, it was demonstrated that exposure to ultrasound significantly increased hGH release. Biocompatibility of the gelatin was determined using both primary human (HOB) and rabbit (ROB) osteoblast-like cells in culture.

1. Introduction

The delivery of growth factors such as hGH can be an important contributing factor in the healing process of bone and the repair of cartilage. It has been shown that hGH can have both direct and indirect effects on osteoblast differentiation and proliferation [1–5] and we have previously demonstrated that hGH can be delivered from bone cement [6, 7], ceramics [8] and other polymer systems [9, 10]. One of the key factors in the design of controlled drug release systems is the choice of an appropriate carrier or vehicle, as this influences the release rate of the incorporated drug. Erodable matrices are controlled by both chemical reactions and diffusion [11, 12], whereas monolithic devices (where the drug is dispersed within the polymer matrix) are osmotically controlled with zero-order drug delivery kinetics [13, 14]. The properties of drug delivery systems can be selected to provide an optimal release rate for the additive, and assure physical and chemical stability of the system. There are numerous polymeric materials available, but only a few have successfully been used for drug delivery [15]. In general, previously studied degradable and non-degradable delivery systems have some limitations; for example, most are able to release only low molecular weight compounds, they display release rates that are either constant or decay with time and their release cannot be modulated once it has commenced [16–19]. Biodegradable polymers are becoming increasingly important in the design of controlled release systems, as they have the major advantage that once the drug has been exhausted they are readily degraded [20, 21]. A variety of biodegradable drug delivery systems have been introduced for controlled drug release and examples include liposomes, gel beads, microcapsules, microspheres and hydrogels [22–26]. In the present study, we have examined the biocompatibility and release kinetics of the natural polymer, gelatin, in microsphere form and as a coating on titanium screws, as a vehicle for the release of hGH. In addition, we have investigated the biocompatibility of the gelatin and examined the effect of ultrasound as a non-invasive method for modifying the amount of hGH released from the microsphere system.

2. Materials and methods

2.1. Preparation of GH-loaded gelatin microspheres

A 20% gelatin solution (300 Bloom, Swine skin type 1) was prepared in sterile water at 37 °C. The solution was divided in two: hGH (8IU) was added to provide a loaded solution to one and the other was a control without hGH. Each solution was then used to prepare microspheres; they were placed in a pre-heated syringe and forced through a 23G needle directly on to ice-cold paraffin oil in a long column, where the microspheres solidified as they collected at the bottom. They were then washed three times in chloroform, cross linked in 25% glutaraldeyhyde vapour, under vacuum, for 48 h and dried in a stream of cool air overnight. Crosslinking of the microspheres was confirmed by a deep yellow colouration; and the mean diameter was 0.4 mm.

2.2. The release of GH from the microspheres

The microspheres were placed in 5 ml phosphate-buffered saline (PBS) and mixed at 37 °C on a continuous rotating mixer. The PBS was changed after 1 h

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and then daily; the eluant was frozen at −20 °C until assayed for immuno- and bioactivity of the released hGH.

2.3. The effect of ultrasound

The microspheres were prepared as described and divided into control and test groups, each comprising 100 mg of microspheres in 5 ml PBS. Those in the test group were exposed to an ultrasonic frequency of 40 kHz (using a DAWE 6441 ultrasonic bath) for 2 min; the PBS was then removed and retained for hGH measurement and replaced. The temperature of the microspheres was monitored and did not exceed 37 °C.

2.4. Preparation of gelatin-coated implants

Commercially pure titanium screw implants, 2 mm × 4 mm, were supplied by Nobelpharma Ltd. and were coated by dipping the screws into a 20% gelatin solution (300 Bloom, Swine skin type 1, Sigma, Poole, UK) containing GH (1 U/ml) and then immediately plunging them into cold water. The gelatin coating was crosslinked in 25% glutaraldehyde vapour, under vacuum, for 6 h and the screws were then placed under a UV lamp overnight. This resulted in an even dry coating of gelatin between the threads of the screws (Fig. 2c). The release of hGH into PBS from the screws was monitored for 14 days to determine the pattern, and total amount of hGH released.

2.5. Effect of pH

The effect of pH on hGH release and degradation was tested for both plain and growth-hormone-loaded microspheres. The swelling kinetics of the microspheres were examined in PBS at pH 2.4, 7.2 and 10.5. 100 mg of dry gelatin microspheres, 28 in number, were placed in 2.5 ml PBS at the appropriate pH. The microspheres were weighed at different time points and the change in weight recorded. The swelling ratios were calculated by comparing the weights of the swollen microspheres to the dry weight of the microspheres with time.

2.6. Assays for growth hormone

An "in-house" ELISA, previously validated to confirm the absence of cross-reactivity and optimized for use with the different elution media [27], was used to measure immunoreactive hGH (antibodies were generously supplied by Novo Nordisk A/S, Gentofte, Denmark).

The bioactivity of the hGH released was measured using an ESTA (eluted stain assay). This cytochemical assay uses NB_2 rat lymphoma cells and relies on the reduction of a tetrazolium salt to a formazan by intracellular dehydrogenase [28].

2.7. Tissue culture

2.7.1. Preparation of gelatin coated tissue culture dishes

Gelatin was prepared as described above, without the addition of hGH, and poured into twelve 35 × 10 mm tissue culture dishes to form a uniform 2 mm layer. The dishes were crosslinked in 25% glutaraldehyde vapour for 48 h.

2.7.2. Biocompatibility

The biocompatibility of the system was investigated using both HOB and ROB osteoblast-like cells. One group of each cell type was seeded (50 000 cells/dish) in Dulbecco's Modified Eagles Medium (Gibco, 10% foetal calf serum) directly onto the gelatin coated dishes. The gelatin-coated titanium implants were placed in a tissue culture dish and seeded with ROB cells (50 000 cells/dish). All materials were maintained in culture at 37 °C, in a humidified atmosphere of 5% CO_2, for a period of 2 weeks. The microspheres were seeded with HOB cells as above and examined using scanning electron microscopy (SEM, Joel JSM 35) 5 days and 2 weeks post-seeding. During this period, all dishes were examined frequently under a light microscope to observe any changes in cellular morphology.

3. Results

3.1. Release of GH from the microspheres and the effect of ultrasound

Both the control and test group readily released high levels of hGH into PBS, with a marked enhancement and significantly more hGH detected in the test group following exposure to ultrasound prior to sampling (students t-test, $p = 0.017$) (Fig. 1a). The hGH released from the microspheres remained bioactive, indicating that it had not been adversely effected (Fig. 1b).

3.2. hGH release from screw fixtures

The hGH-loaded gelatin coated screws, were able to release hGH *in vitro*, with the bulk elution occurring during the first 4 h. This was followed by a much slower continuous release for up to 5 days. The rate at which the hGH is released is dependent on the thickness of the coating and the extent of crosslinking (Fig. 1c).

3.3. Effect of pH

The swelling kinetics of both the control and hGH-loaded microspheres were affected by pH. During the first 3 h, the microspheres swelled in all cases, resulting in an increase in weight and swelling ratio. This initial increase was followed by a marked fall in the swelling ratio of those microspheres at pH 2.4 and pH 10.5, at approximately 9 days, with only a small drop observed for the microspheres at pH 7.2. These findings were consistent for both hGH-loaded (Fig. 1d) and the control microspheres.

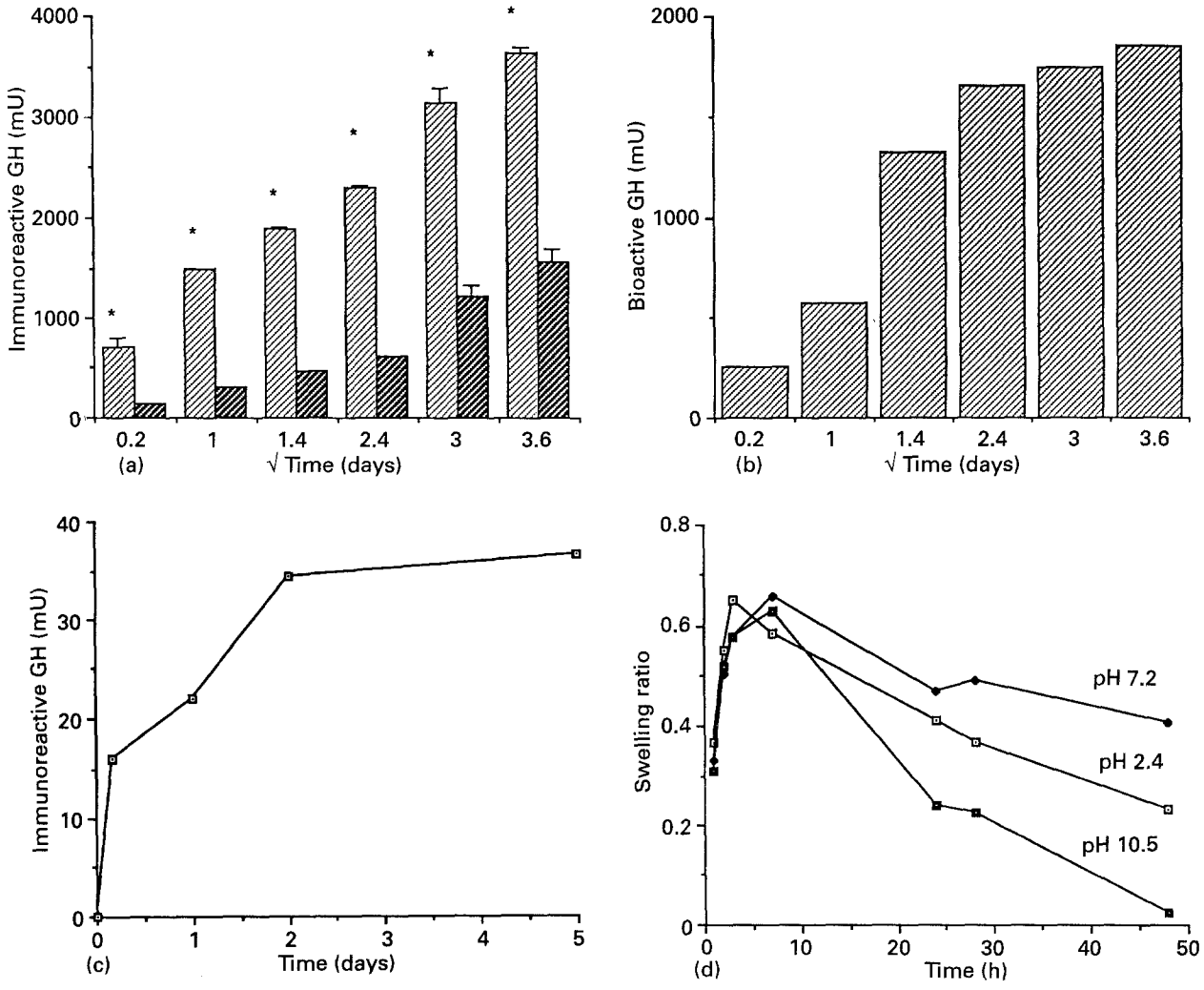

Figure 1 (a) The release of GH from the microspheres into PBS in the test group exposed to ultrasound (▨), and in the control group (▨). A significant (*) increase in the amount of GH released was seen in the test group exposed to ultrasound ($p = 0.007$). (b) The release of bioactive hGH from gelatin microspheres. (c) The *in vitro* release of hGH from gelatin-coated titanium screw fixtures. (d) The effect of pH on the swelling kinetics of the hGH-loaded microspheres. A rapid rise in the swelling ratio was seen during the first 3 h. The rate of hydrolysis was much faster at pH 2.4 and 10.5.

3.4. Biocompatibility

The gelatin-coated tissue culture plates supported growth of both human and rabbit osteoblast-like cells (Fig. 2a and b). In the 48 h crosslinked dishes, HOB cells grew to confluency and although there was visible evidence of degradation of the gelatin after 21 days in culture, the cells retained their typical morphology and appeared unaffected by any degradation products. The ROB cells grew rapidly on the gelatin coated screws, with a large number around the screw threads. Once again there was no evidence that even after 14 days in culture, degradation products affected cell growth (Fig. 2d).

Scanning electron microscopy (SEM) showed that HOB cells grew well on the microspheres, and after several days in culture the cells appeared to infiltrate the gelatin matrix (Fig. 3a). Areas of degradation were evident around the cells 2 weeks after seeding; probably a direct effect of enzymatic degradation by the cells (Fig. 3b).

4. Discussion

Gelatin is a natural biocompatible polymer, and we have shown that gelatin can be used as a vehicle for the delivery of hGH in microsphere form and as a coating. The biological environment in which the delivery system is to be used will have an effect on both the rate and the amount of the additive released. Gelatin has a setting temperature of 35 °C and pH limits between 3 and 10, thus making it suitable for the incorporation and delivery of hGH and other growth factors that may be susceptible to damage by excessive changes in temperature and pH [29–32]. Although the solubility of gelatin in water may appear to be a disadvantage with regard to the rate of hydrolysis, careful manipulation of the preparation conditions and crosslinking time results in minimal destruction of the gelatin molecule [33]. Water plays an important role in the biodegradation rate of polymer-based drug-delivery systems, the solubility of gelatin in water makes it ideal for the absorption of tissue fluids and proteins from the surrounding bone matrix and marrow. Gelatin microspheres and coatings could be used to release therapeutic doses of growth factors to a target site, and have the further advantage that once the drug is released they are degraded. The mode of release of hGH from the microspheres has previously been described, and is partially due to diffusion;

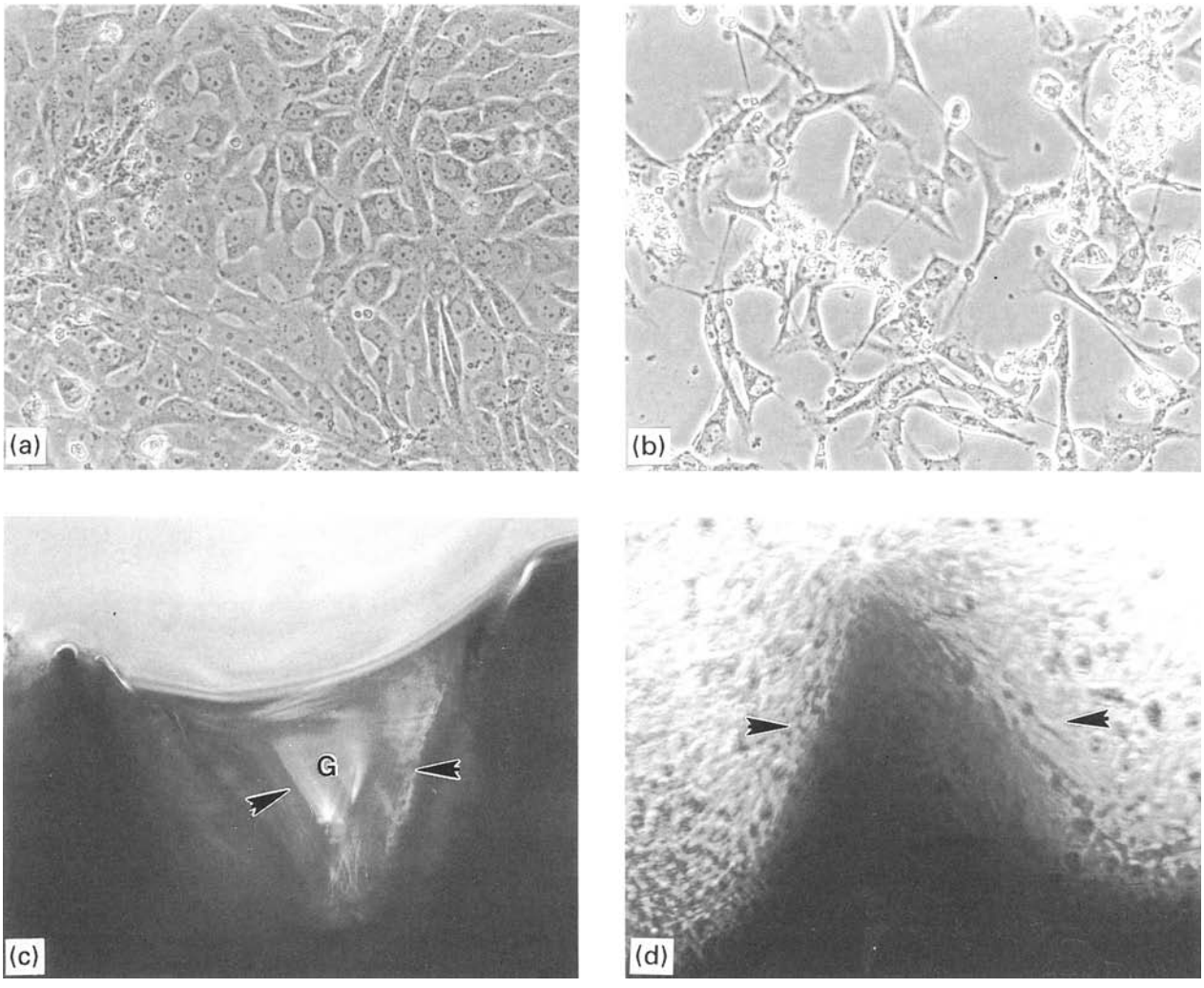

Figure 2 (a) Phase contrast (× 200) of HOB cells on gelatin-coated tissue culture plastic, the cells grew to confluency and retained their morphology. (b) Phase contrast (× 200) of ROB cells growing on gelatin-coated tissue culture plates, the cells appeared more elongated and approaching confluency. (c) Magnified view (× 200) of a gelatin-coated screw, the gelatin (G) lying mainly within the screw thread (arrows) of the fixture. (d) Phase contrast (× 200) of ROB cells growing on the gelatin-coated screws, cells can be seen growing between the screw threads (arrows).

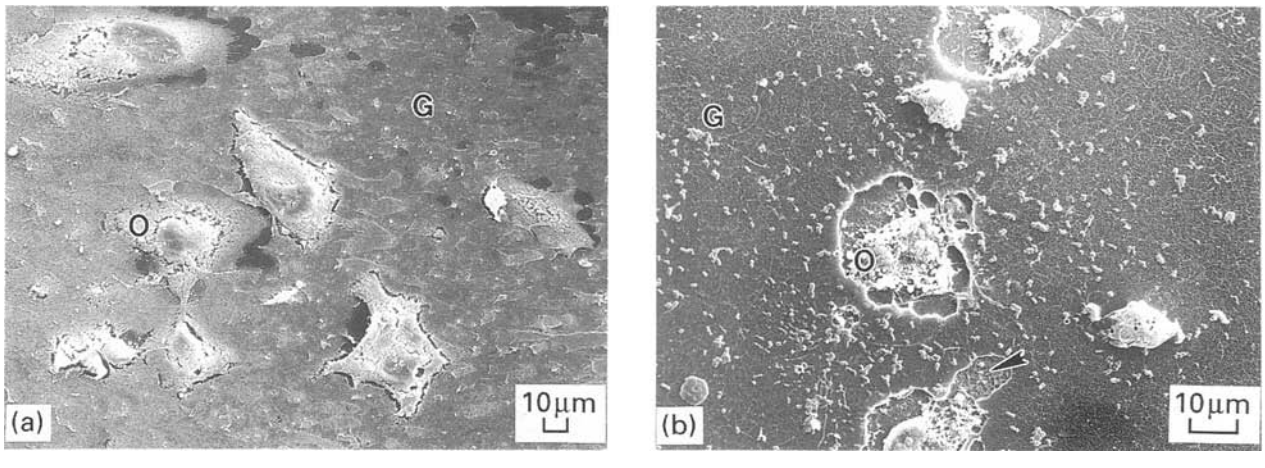

Figure 3 (a) SEM showing the adherence of HOB (O) cells on the gelatin microsphere (G). (b) SEM of HOB (O) cells 2 weeks post-seeding onto gelatin microsphere (G); areas of degradation were visible around the cells which had infiltrated the gelatin matrix (arrow).

followed by a second phase which is controlled by the rate of degradation, and the extent of the initial crosslinking of the gelatin [34]. Our results show that hGH can be released from the microspheres, with a twofold increase in the amount of hGH released following exposure to ultrasound. One possible explanation for the enhancement seen, is that cavitation, induced by the ultrasonic waves results in an increase

in the penetration of water, thereby promoting hydrolytic degradation and the release of more hGH. This type of modulation may prove useful for the release and efficacy of peptides such as hGH, by allowing a more subtle and physiological delivery pattern. Kost *et al.* have demonstrated that ultrasound affects the release rates of implantable controlled delivery devices [35, 36].

The rate of cell proliferation and bone formation at a trauma site could be improved by the release of growth factors locally. In general, these factors have a short biological half-life, and their actions are mediated through autocrine and/or paracrine pathways from locally stimulated cells [37]. An increase in local bioavailability could be of potential use in the treatment of bone defects and disease where rapid tissue regeneration and wound healing is desirable. The microspheres could be used directly at the bone-implant site, for example for filling bone defects, or they could be incorporated into allograft material to stimulate tissue regeneration. The advantage of this system is, that it is degraded once the drug release has been exhausted. Coatings on metals and other materials have been used to improve bonding at bone and soft tissue interfaces [38–40]. We have demonstrated that gelatin can be used to successfully coat implants such as titanium screw fixtures. The biodegradable, biocompatible gelatin polymer that we have described could have numerous clinical applications, both in the form of microspheres and as a coating on prostheses.

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