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Hypromellose films for the delivery of growth factors for wound healing

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Abstract

Hypromellose was investigated as a carrier for extending topical growth factor delivery to wounds. Films of hypromellose (E4M, K4M and E10M) containing a model protein horseradish peroxidase (1% w/w HRP, MW 40 000) were cast from aqueous solutions and dried at 37°C. In-vitro release was determined using Franz-type diffusion cells and films were mounted directly into the Franz cell or cast onto a wound dressing (Melolin) backing. There was an initial burst release then an extended release over 5 h. The Melolin backing significantly reduced the burst but not the extended release rates ($P < 0.05$). Release of HRP was also determined from 7% w/v hypromellose gels and was significantly lower for E10M than E4M, suggesting that, once hydrated, the E10M hypromellose provides the greatest resistance to HRP release. The release profile of basic fibroblast growth factor from Melolin-backed films made from E4M hypromellose was not significantly different at any time point to that of HRP release from the same formulation. Hypromellose may be incorporated into a wound dressing such as Melolin to provide a prolonged release of an incorporated protein active.

Introduction

Healing of some chronic ulcers has been suggested to be hindered by a relative lack of growth factors when compared with acute wounds (Bennett & Schultz 1993a; Falanga 1993; Colville-Nash & Willoughby 1997; Harding et al 2002; Martin et al 2003). To correct this deficiency, topically applied growth factors have been studied (Bennett & Schultz 1993a; Stadelmann et al 1998). In the wound environment topically applied agents, even protein compounds, may be able to penetrate through the wound tissue because the integrity of the stratum corneum is lost. However, there are still several obstacles to overcome to achieve optimal sustained delivery via the topical route. These include a general lack of information about the most appropriate growth factor to apply and the disposition of growth factors in the wound following administration. Typically, growth factors, such as basic fibroblast growth factor and vascular endothelial growth factor, have short half-lives in-vivo. In the wound they are subjected to rapid degradation (Yerushalmi et al 1994; Bennett et al 2003) due to the presence of high protease activity especially in chronic wounds (Tarnuzzer & Schultz 1996; Trengove et al 2000; Harding et al 2002). In these chronic wound conditions, growth factors are either present at too low concentrations or are at effective concentrations for only short periods and need to be protected from premature clearance (Robertson et al 1999; Culajay et al 2000). Hence, sustained-release formulations are being investigated (Kawai et al 2000). Currently there is one commercial product, Regranex Gel, which contains a recombinant human platelet-derived growth factor at a concentration of 0.01% w/w in a sodium carboxymethylcellulose gel.

Hypromellose (hydroxypropylmethylcellulose) has been used in sustained-release pharmaceutical products for many years (Alderman 1984; Sung et al 1996). When used in matrix tablets, these polymers hydrate on contact with water to produce a viscous gel barrier within and surrounding the tablet (Mitchell et al 1990). The properties of hypromellose that affect the rate of drug release include the rate of diffusion of water into the dry polymer, the rate of hypromellose hydration and gel formation, the viscosity of the hydrated hypromellose and rate of hypromellose gel erosion (Duddu et al 1993). Since most growth factors are high-molecular-weight compounds (e.g. fibroblast growth factor, MW 16 000; vascular endothelial growth factor, MW 45 000) (Bennett & Schultz 1993b), the viscous gel formed by

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hydrated hypromellose may represent a sufficient diffusional barrier to slow the rate of delivery of growth factor from a topically applied gel to an underlying wound.

Several grades of hypromellose are available and these differ according to the type (methoxyl or hydroxypropyl) and degree of substitution of the cellulose backbone (Whelan et al 2002). To evaluate the use of hypromellose polymers in providing a prolonged-release system for growth factors, we report the release of a model protein, horseradish peroxidase, and a model growth factor, basic fibroblast growth factor (bFGF), from dried films, films prepared on Melolin wound dressings and preformed gels.

Materials and Methods

Materials

Hypromellose (Methocel) E4M CR, K4M CR and E10M CR Premium EP were from Colorcon (Kent, UK). Degrees of substitution for these hypromellose polymers were: 1.9 methoxyl and 0.23 hydroxypropyl for E4M and E10M and 1.4 methoxyl and 0.21 hydroxypropyl for K4M. The viscosity of 2% w/v hypromellose in water at 20°C is 4000 mPa s, 10 000 mPa s and 4000 mPa s for E4M, E10M and K4M, respectively. Horseradish peroxisade (HRP; Type I, EC 1.11.1.7) was obtained from Sigma-Aldrich Pty Ltd (Sydney, Australia). Recombinant human basic fibroblast growth factor (rhbFGF derived from *Escherichia coli*) was obtained from RnD Systems (Minneapolis, USA). Melolin was a gift from Smith and Nephew Limited (Auckland, New Zealand). All other chemicals were analytical grade purchased from BDH Chemicals New Zealand Ltd (Palmerston North, New Zealand).

Effect of temperature on HRP activity

HRP activity in solution at elevated temperatures (4–85°C) was determined. HRP ($100\,\mu g\,mL^{-1}$) solutions ($n=3$) were incubated in a water bath at elevated temperatures for either 2 or 10 min, and then enzyme activity was determined using a method described by Chance (1943) with pyrogallol as a substrate. Results were expressed as specific enzyme activity relative to control solutions kept on ice.

Preparation of HRP containing hypromellose films and gels (E4M CR, E10M CR or K4M CR hypromellose)

Isotonic phosphate-buffered saline (PBS) was prepared containing 13.6 mM KH_2PO_4, 6.4 mM Na_2HPO_4 and 121 mM NaCl at pH 6.5.

Hypromellose (0.1 g) was added slowly to 5 mL PBS at 80°C with stirring. HRP (0.001 g) was dissolved in a further 5 mL of PBS at room temperature, and then added to the hypromellose solution at <40°C. Resulting solutions were stirred continuously for 2 h at room temperature. Portions (150 μL) of the prepared hypromellose/HRP solutions were transferred to either non-adherent Melolin backing material (diameter 1.5 cm) placed on a Parafilm template or directly onto a Parafilm template. These were placed in an incubator (Contherm Biocell 1000) at 37°C and allowed to dry overnight. Once dried, films were peeled off the Parafilm templates and contained a theoretical loading of 1% w/w HRP in hypromellose.

Hypromellose gels were prepared in PBS as described above for films except that the final concentration was 7% w/v hypromellose with 0.07% w/v HRP. These were used without drying in release experiments to determine release of HRP from hydrated hypromellose. At a concentration of 7% w/v, the hypromellose gels had sufficient viscosity so that they remained within the donor compartment of the Franz cell during the release experiments. The ratio of HRP to hypromellose was 1:100 w/w.

Preparation of bFGF-containing films (E4M CR hypromellose)

Dried films containing 0.2% w/w bFGF in hypromellose were prepared on the Melolin backing as described above for HRP.

In-vitro release of HRP from films and gels

Modified Franz-type diffusion cells (diameter 1.4 cm, receptor volume 4 mL), water-jacketed and maintained at 37°C, were used. PBS (pH 6.5) was used as the release medium for all experiments. Hypromellose films were removed from the Parafilm backing and then mounted in the Franz-type cells. Gels (1.5 g) were filled into the donor compartment of the Franz cells. At times 1, 2, 3, 4, 5, 10, 30, 60, 120, 180, 240 and 300 min, samples (2 mL) were removed and replaced with fresh pre-warmed buffer. HRP released was quantified by size-exclusion high-performance liquid chromatography (SEC-HPLC).

The HPLC system (Shimadzu, Kyoto, Japan) comprised an LC 10-AT pump, a SIL-10AD auto-sampler injector (injection volume 100 μL), and an SPD-10A variable wavelength detector (set at 220 nm) controlled by a computer using Class-VP 6.1 software. The stationary phase was a Biosep-SEC-S 2000, 300×7.8 mm i.d. column (Phenomenex, New Zealand). The mobile phase was 20 mM Na_2HPO_4 and 150 mM NaCl, pH 6.8 at a flow rate of 0.8 mL min^{-1}. The calibration curve for HRP was linear over the range 10–150 $\mu g\,mL^{-1}$ ($R^2>0.99$). Control experiments showed that dissolved hypromellose did not interfere with the assay for HRP.

GraphPad Prism (version 4.01 for Windows, GraphPad Software, San Diego, CA) was used to fit linear equations to the initial (0–5 min) and extended release (1–5 h) periods.

In-vitro release of bFGF from E4M CR hypromellose films with Melolin backing

Release was followed as for HRP, except sampling times were 10, 30, 60, 120, 180, 240 and 300 min. The released bFGF was quantified using a Quantikine, human FGF basic immunoassay kit (RnD Systems, Minneapolis, USA). This

assay was linear over the bFGF concentration range 20–640 pg mL^{-1} ($R^2 > 0.99$).

Results

Effect of temperature on HRP activity

The percentage HRP activity retained upon heating is shown in Figure 1. There was no significant activity loss until a temperature of 70°C was reached. Maintaining the solution at a temperature of 80°C for 10 min resulted in greater than 50% loss of activity. For this reason, HRP was added to hypromellose solutions at temperatures below 40°C.

In-vitro release from hypromellose films and pre-formed gels

Cumulative release profiles for HRP released from hypromellose films and Melolin-backed hypromellose films are shown in Figures 2A and 2B, respectively. The release profiles did not fit either a zero- or first-order model but could be described with linear models over an initial period (1–5 min) and an extended period (60–300 min) (Figure 3). Table 1 shows the rates (%/min) of HRP release over these periods and the percentages of HRP released at 5 and 300 min.

Analysis of variance of release rates showed that Melolin film backing significantly reduced the HRP release rate during the initial, but not the extended, release period ($P < 0.05$). The hypromellose grade did not significantly affect the release rates from either the Melolin-backed films or non-backed films ($P > 0.05$). The amount released at 300 min was significantly lower for K4M hypromellose from the non-backed films only ($P < 0.05$).

From the pre-formed gels (Figure 4), HRP was released significantly more slowly from the E10M hypromellose than either E4M or K4M. Very little difference was observed between E4M and K4M, especially over the first 2 h.

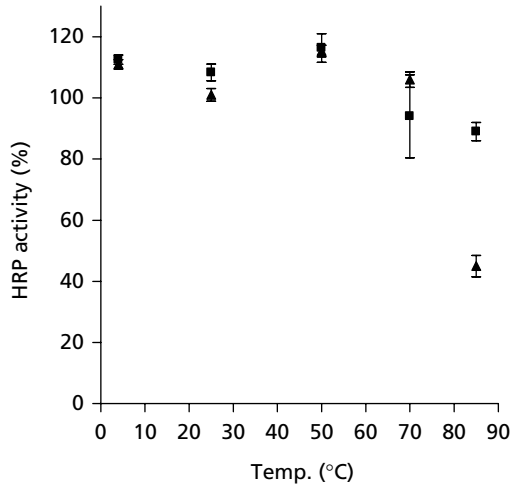

Figure 1 Effect of temperature on HRP enzyme activity in solution after 2 (squares) or 10 (triangles) min incubation. Means of triplicates are plotted and error bars represent s.e.m., n = 3.

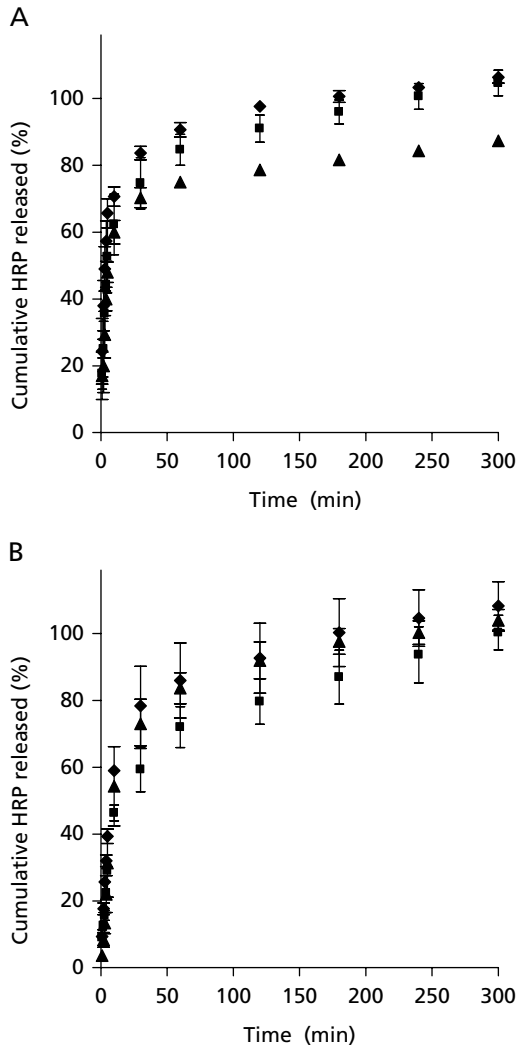

Figure 2 Cumulative HRP release from E4M (squares), K4M (triangles) and E10M (diamonds) hypromellose films (A) and Melolin-backed films (B). Means of triplicates are plotted and error bars represent the s.e.m., n = 3.

In-vitro release of bFGF from E4M CR hypromellose films with Melolin backing

Cumulative release of HRP and bFGF from E4M CR hypromellose on the Melolin backing is shown in Figure 5. Repeated measures analysis of variance of the percentage released (HRP or bFGF) at each time point showed no significant difference between the two proteins. The mean extended release rates (± s.e.m., n = 3) were 0.08 ± 0.01 % $cm^{-2}\,min^{-1}$ and 0.09 ± 0.03 % $cm^{-2}\,min^{-1}$ for HRP and bFGF, respectively, from these Melolin-backed films ($P < 0.05$).

Discussion

Melolin (Smith & Nephew, Hull, UK) is a low-adherent absorbent wound dressing composed of three layers: a thin perforated polyester film, a highly absorbent cotton/polyester

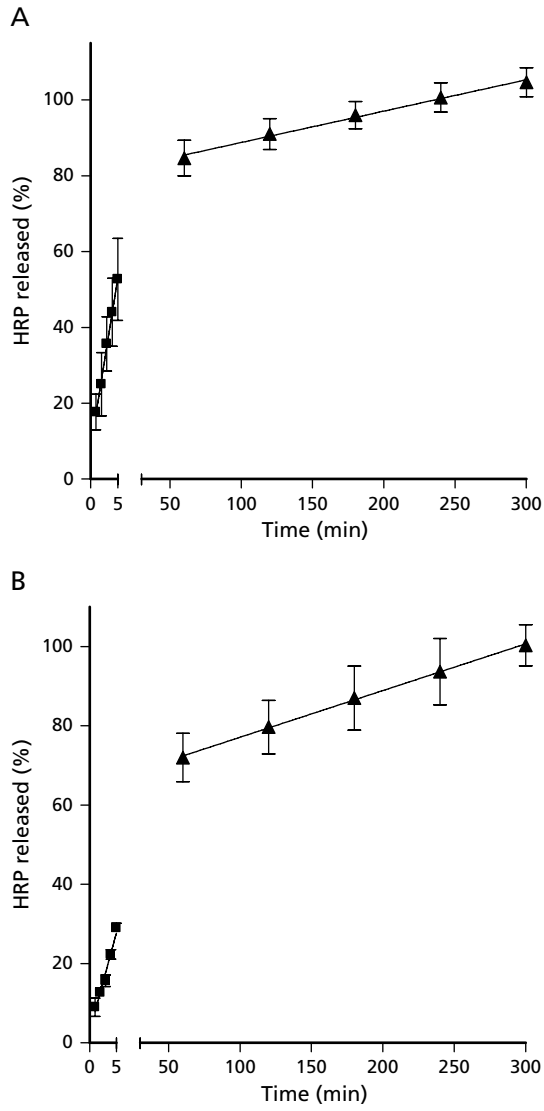

Figure 3 Initial and extended release of HRP from E4M CR hypromellose films (A) and Melolin-backed films (B). Means of triplicates are plotted and error bars represent the s.e.m., n = 3.

pad and a hydrophobic outer backing layer. To investigate the use of this material as a platform for the administration of a prolonged-release system for growth factors to wounds, the polyester film was carefully removed from the cotton layer and hypromellose containing a model protein (HRP) was added by drying a solution onto the film surface. By incorporating a growth factor into hypromellose on a wound dressing it is proposed that slow hydration of the polymer on contact with the wound fluid will slowly produce a gel from which the growth factor will be released over an extended period. The initial results reported in this paper sought to characterise the effects of some selected formulation variables on the release of a model protein. HRP was chosen as the model protein for study because its concentration could be analysed using SEC-HPLC and enzyme activity could be monitored as a marker of retained protein activity with formulation.

Release of compounds from dried hypromellose films was expected to be dependent on the rate of hypromellose hydration and the formation and stability of the gel layer that subsequently forms (Kiortsis et al 2005). Diffusion of a compound through the surface gel and dissolution or erosion of the gel were both expected to contribute to compound release (Mitchell et al 1993; Kim & Fassihi 1997). Several equations have been described in the literature to model the release of drugs from hypromellose matrices, including zero-order, square-root time and a power law equation (Kiortsis et al 2005). These models are fitted to around 60% of the drug release (Peppas 1985; Rinaki et al 2003), although Rinaki et al (2003) have shown that the power law equation could be applied to the entire release profile for some hypromellose-based matrix tablets. These models did not fit our data, but the data could be described by linear models fitted to an initial burst (0–5 min) and a period of extended release (1–5 h, $R^2 > 0.90$).

The different hypromellose grades have different hydration rates and gel viscosities (Alderman 1984). E4M and K4M have equal viscosities at concentrations of 2% w/v in water, although the K4M, which has a lower degree of methoxyl substitution, is reported to hydrate more rapidly (Alderman 1984). This may be important, as a polymer must hydrate quickly enough to form a gel layer before the active can dissolve prematurely (Alderman 1984). The more rapid release of HRP from E4M compared with K4M hypromellose appears consistent with this. E10M has an equivalent degree of substitution of methoxyl and hydroxypropyl but a higher viscosity at 2% w/v in water than the E4M polymer. Comparison of HRP release from the preformed gels shows the rate and extent of release of HRP was slower and lower for E10M compared with E4M, which is consistent with the greater diffusional barrier produced by the higher-molecular-weight

Table 1 Summary of least squares linear regression for HRP release

Grade	Melolin	Initial release (0–5 min)			Extended release (60–300 min)		
		Slope (% min^{-1})	% released at 5 min	R^2	Slope (% min^{-1})	% released at 300 min	R^2
E4M	–	8.9 ± 2.5	52.7 ± 18.7	> 0.95	0.08 ± 0.01	104.7 ± 6.7	> 0.98
	+	5.0 ± 0.7	29.0 ± 2.0	> 0.90	0.12 ± 0.03	100.3 ± 9.0	> 0.91
K4M	–	8.2 ± 2.8	48.0 ± 5.3	> 0.93	0.05 ± 0.02	87.3 ± 1.5	> 0.99
	+	6.9 ± 4.2	31.3 ± 17.6	> 0.96	0.08 ± 0.01	104.0 ± 5.6	> 0.91
E10M	–	10.2 ± 2.5	65.7 ± 7.5	> 0.96	0.06 ± 0.01	106.3 ± 2.9	> 0.93
	+	7.4 ± 0.6	39.3 ± 3.8	> 0.99	0.09 ± 0.04	108.3 ± 12.6	> 0.94

Values are mean ± s.e., n = 3.

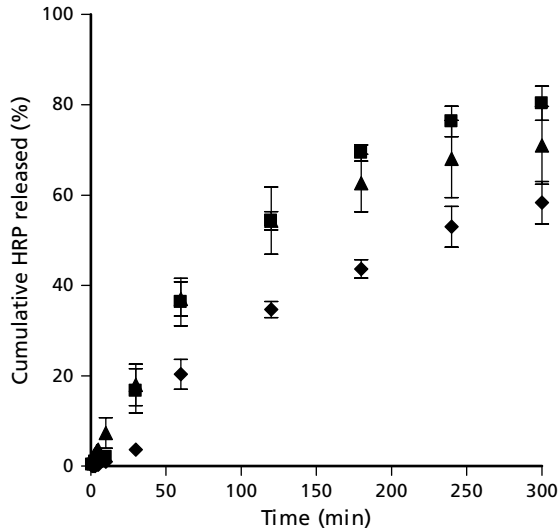

Figure 4 Cumulative HRP release from pre-formed gels E4M (squares), K4M (triangles) and E10M (diamonds) hypromellose. Means of triplicates are plotted and error bars represent the s.e.m., n = 3.

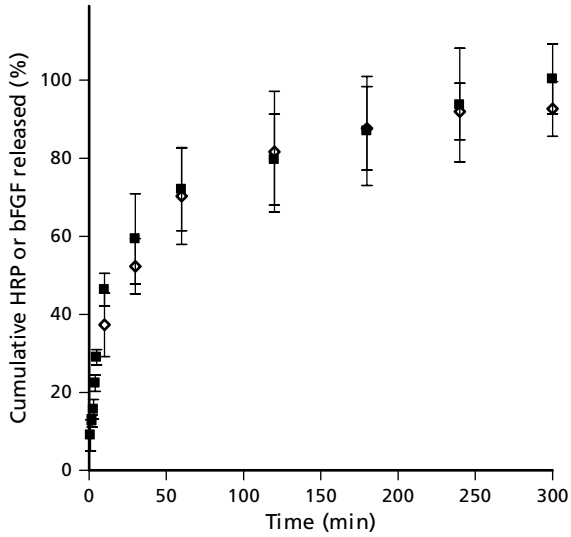

Figure 5 Cumulative bFGF (diamonds) and HRP (squares) release from E4M CR Melolin-backed films. Means of triplicates are plotted and error bars represent the s.e.m., n = 3.

hypromellose. Similarly, Sung et al (1996) reported a lower release of their model drug (adinazolam mesylate) from the higher-viscosity K15M grade when compared with the K4M hypromellose polymer. To test the hypothesis that hydration rate led to the difference in HRP release between the E4M and K4M polymers, release from preformed gels was studied (Figure 4). With preformed gels, E10M slowed the release (Figure 4), whereas for films, K4M slowed release more than E10M (Figure 2A). This may be due to rapid hydration forming a diffusional barrier faster and retarding the movement of the protein from the gel. The concentration of hypromellose used was 7% w/v because this produced a gel of sufficient viscosity to have a semi-solid consistency and it did not flow from the donor compartment of the Franz-type diffusion cell. The statistically significant difference between E10M and E4M from the pre-formed gels indicates that once the gel layer is formed it is expected that the E4M polymer produces the lowest resistance to HRP release, while the higher-molecular-weight E10M polymer produces the greatest. The difference in release rates between E4M and K4M preformed gels was not significant, suggesting that once the gel is formed these polymers offer similar resistances to HRP release.

Preparation of the hypromellose films on Melolin backing reduced the initial burst release rate. This effect may have been due to the increased hypromellose film integrity gained by the backing material. This may be important in the delivery of growth factors to wounds using gel-based vehicles because the initial delivery of growth factor into the wound may be slowed if it is delivered from a wound dressing platform compared with direct application of a gel solution to the wound surface. It is anticipated that the initial release would replenish wound levels of the applied factor to levels consistent with improved wound healing, and then the extended release would maintain factor levels in the wound until the wound dressing is changed. Further work in defining the ideal release profile for topically applied growth factors in wound healing is needed. Current literature for fibroblast growth factor suggests it plays an important role in the initial stages of wound healing (Nissen et al 1996), but needs to be delivered in a sustained-release formulation to compensate for its short biological half-life (Kawai et al 2000). Dinbergs et al (1996) reported that sustained release of bFGF from alginate/heparin sepharose microspheres gave a 3-fold increase in vascular endothelial and smooth muscle cell proliferation in culture compared with a bolus application. This formulation releases bFGF over a 5-h period, which is expected to be long enough for target cells to commit (e.g., EGF needs a prolonged contact time of 4 h) (Aharonov et al 1978). Work by Cross & Roberts (1999) reports that the topical permeability of a wound to growth factors decreases as the wound heals; therefore, it may be expected that release from the topically applied system is most important in the early applications and transfer across the healing wound becomes important for latter applications of growth factors.

Figure 5 shows that the release of bFGF and HRP from Melolin-backed hypromellose films are not statistically different. This suggests HRP may indeed be an appropriate model for in-vitro investigation of bFGF release from hypromellose films. The period of extended release of bFGF over 300 min (or 5 h) suggests that the hypromellose films on a Melolin backing may represent a useful carrier for delivery of this growth factor to wounds. Further studies are planned to assess the duration of delivery of bFGF in-vivo.

Conclusions

Hypromellose polymers may have potential as prolonged-release vehicles for the topical delivery of growth factors to wounds. By drying hypromellose gels onto a wound dressing, such as Melolin, the period of delivery of incorporated agents

may be prolonged as film integrity appears to be retained in an aqueous environment.

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