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Carbohydrate Polymers 60 (2005) 523–528
Carbohydrate Polymers
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IPNs based on chitosan with NVP and NVP/HEMA synthesised through photoinitiator-free photopolymerisation technique for biomedical applications

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Received 3 December 2004; revised 4 March 2005; accepted 15 March 2005
Available online 29 April 2005

Abstract

Biocompatible interpenetration polymeric network (IPN) hydrogels based on chitosan with *N*-vinylpyrrolidinone (NVP) as well as its copolymer with 2-hydroxymethyl methacrylate (HEMA) were synthesised using the photopolymerisation technique without the inclusion of any photoinitiator or crosslinking agent. These hydrogels were characterised using the Fourier-transform infrared spectroscopy (FTIR) technique. Equilibrium swelling of these hydrogels was performed in Milli-Q water and drug release studies were carried out using theophylline as the model drug. These tests showed that the IPN comprised of chitosan and NVP with a very small amount of *N*-hydroxymethyl maleimide (HMMI) included exhibited higher swelling abilities and fast drug release rates than the IPN which contained chitosan, NVP and HEMA. Kinetic studies of water diffusion into these hydrogels and drug release revealed that with the exception of the IPN with HEMA incorporated, the other hydrogels did not adhere to the Fickian diffusion model. These hydrogels were tested for their biocompatibility with human epidermal keratinocyte cells (HaCaT). A positive cell growth as evidenced by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay indicated that these hydrogels are non-toxic to human keratinocytes and can be potentially used as biomaterials for biomedical applications.

Keywords: Chitosan; IPN hydrogel; Photopolymerisation; Keratinocytes; Biomaterial

1. Introduction

Chitin, extracted from crustacean shells, is a biopolymer consisting of β-(1-4)-2-acetamido-2-deoxy-D-glucose units and chitosan is obtained from chitin by a deacetylation process. Chitosan is a weak base with a pK_a value of 6.2 (Hejazi & Amiji, 2002), and therefore is insoluble in neutral and alkaline environments. This property thus contributes to its non-swelling nature in water. However, chitosan has the inherent properties of being non-toxic, biodegradable, and biocompatible (Singh & Ray, 1994) and hence, make it a promising matrix in the preparation of interpenetrating polymer network hydrogel, a complex polymer composed of two kinds of polymers, for biomedical applications. IPN hydrogels involving chitosan have been reported for biomedical applications including wound dressings (Risbud, Hardikar, Bhat, & Bhonde, 2000), drug delivery (Shu, Zhu, & Song, 2001) and fabrication of biosensors (Ng, Guthrie, Yuan, & Zhao, 2001).

In this study, IPNs were synthesised by allowing NVP (an electron donor) in the presence of a small amount (4%, w/w) of HMMI (an acceptor) as well as a mixture of NVP/HEMA to polymerise in chitosan solutions using the photopolymerisation technique without the inclusion of any photoinitiator. This is the first time that IPNs based on chitosan with these monomers using this technique have been reported. This technique is unique, as a photoinitiator (PI), which is normally needed to initiate a photopolymerisation process, is not required in this synthesis. The use of PIs is a growing concern with regards to their incomplete usage in the polymerisation process. This may result in undesirable impurities, which

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doi:10.1016/j.carbpol.2005.03.009

exist as unreacted fragments formed from their photolysis process. These 'leachable' small fragments being embedded in the polymer matrices may leach out to the environment with time.

In this work, the photopolymerisation of NVP within the chitosan matrix was, however, initiated by a donor/acceptor pair with NVP as the donor and HMMI and HEMA as the acceptors, under the influence of a UV source. HMMI and HEMA each possesses an electron withdrawing group adjacent to the double bond in their structures thus contributing to the electron accepting property in these monomers. The mechanism for the initiation of the photopolymerisation process involving a similar donor/acceptor pair has been previously reported (Ng, Jonsson, Swami, & Lindgren, 2002). Essentially, this mechanism involves strong interactions between a donor and acceptor when both species are being exposed to a UV source leading to the formation of an intermediate. This intermediate species then breaks down to form free radicals, which initiate polymerisation. A significant advantage of using a donor/acceptor pair in the initiation process is that it has the dual function of acting as an initiator as well as forming a copolymer. FTIR was the technique employed to confirm the presence of various components in the IPN hydrogel structures.

Swelling behaviours of these hydrogels were evaluated in Milli-Q water and the abilities of these IPN hydrogels in controlled-release using the model drug, theophylline, were investigated. Spectrophotometry was the technique exploited in this study for the quantitative measurement of drug release.

Since hydrogels find applications in biological systems, it is therefore essential that they do not have toxic effects on these systems. In view of this, toxicity test should be an important aspect of research in biomaterials. Accordingly, the hydrogels understudy were tested in vitro for their biocompatibility with human epidermal keratinocyte (HaCaT) cells. In the panorama of numerous established cell lines, HaCaT has a very interesting feature, having a close similarity in the functional competence to that of normal keratinocytes (Pessina et al., 2001). This cell line has been used in numerous studies as a paradigm for epidermal cells. Furthermore, it is readily available, highly sensitive and easily regenerated, thus making it an ideal candidate as the cell model for this work.

A MTT assay, which is a colorimetric based technique for non-radioactive quantification of cellular proliferation, viability, and cytotoxicity was used for this work. The assay is based on the cleavage of the yellow tetrazolium salt (MTT) to form purple formazan crystals by dehydrogenase activity in active mitochondria, which indicate the presence of live cells (Gerlier & Thomasset, 1986; Mosmann, 1983).

For comparative purposes, toxicity test was also performed on the monomers, NVP, HMMI, and HEMA.

2. Materials and methods

2.1. Materials

NVP, HEMA, chitosan (85% deacetylated), MTT, theophylline, and maleimide, the reagent required for the synthesis of the HMMI, were purchased from Aldrich, NSW, Australia. These reagents were used as received with the exceptions of NVP, which was purified by vacuum distillation and HEMA was purified by passing through an inhibitor remover pre-packed column supplied by Aldrich to remove the stabiliser, hydroquinone. HaCaT cells were provided by the Skin Technologies Research Centre, UWS. Ca^{2+} free Dulbecco's modified Eagle's medium was supplied by Thermo Trace, Victoria, Australia, and fetal calf (bovine) serum (FCS) was purchased from JRH Biosciences, Victoria, Australia.

2.2. Methods

2.2.1. Synthesis of HMMI

HMMI was prepared using the method as outlined by Tawney et al. (1961). Maleimide in the presence of formaldehyde was heated to 35 °C after which a dilute NaOH solution was added. The solution was stirred for a further 2 h, cooled to room temperature and then placed in a freezer overnight. The resulting white crystals as HMMI were then collected by filtration. The HMMI thus obtained was further purified by sublimation to yield white crystals and their purity was evaluated using NMR spectroscopy (Varian, 400 MHz).

^{1}H NMR (D_2O, δ ppm): 6.79 (d, 2H, –CH=CH–, aromatic); 4.88 (s, 2H, –N–CH_2–O–). ^{13}C NMR (D_2O, δ ppm): 172.26 (O=C–N); 135.31 (C=C); 59.73 (N–C–OH).

2.2.2. Hydrogel preparation

Chitosan solution (CS) (1.15%, w/v) was prepared by dissolving an appropriate quantity of chitosan in 0.9 M levulinic acid. Solutions comprising of CS:NVP:HMMI in the ratio of 33.5:62.3:4.2% (w/w), respectively (Formulation (i)) as in Table 1; CS:NVP:HMMI in the ratios of

Table 1
Polymerisation status of formulations with chitosan pre-dissolved in levulinic acid

IPN formulation (%, w/w)	Polymerisation status upon exposing to a UV source
i) CS (33.6%)–NVP (62.4%)–HMMI (4.0%)	Partially opaque IPN formed
ii) CS (48%)–NVP (48%)–HMMI (4.0%)	Partially opaque IPN formed
iii) CS (62.4%)–NVP (33.6%)–HMMI (4.0%)	DNP
iv) CS (50%)–HEMA (50%)	DNP
v) CS (50%)–NVP (25%)–HEMA (25%)	Clear and pale yellow IPN formed

Conditions: UV dose, 9 kJ; temperature, ambient temperature; DNP, did not polymerise.

48.0:48.0:4.0% (w/w) and 62.4:33.6:4.0% (w/w) for Formulations (ii) and (iii), respectively; CS:HEMA in the ratio of 50.0:50.0% (w/w) for Formulation (iv) and CS:NVP:HEMA in the ratio of 50:25:25% (w/w), respectively (Formulation (v)) were prepared.

The reacting solutions (1.0 ml) were each placed in a non-pigmented polypropylene mould with a bore diameter of 0.5 cm and subjected to UV exposure with a 90 W medium pressure mercury lamp model $93110E_2$ mounted in a vertical configuration. All experiments were performed at room temperature at a distance of 30 cm from the radiation source with a peak irradiance of 0.80 mW/cm^2 as measured by a 1L390A curing radiometer from International Light. These preliminary trials showed Formulations (i) and (v) produced the optimum hydrogels (Gels A and B, respectively) that were resilient and had high water content after swelling.

2.2.3. Swelling test

Procedures were similar to those outlined in a previous publication (Ng et al., 2002). However, before the swelling test, the samples were repeatedly washed in Milli-Q water to remove unreacted monomers and the washing continued until less than 1 ppm of the unreacted monomers were present in the washed solution, detected using the spectrophotometric technique. The swollen masses of the hydrogels were then compared with their dry masses in order to calculate the equilibrium water content (EWC) according to Eq. (1)

$$\mathrm{EWC}(\%) = \frac{W_t - W_0}{W_t} \times 100 \quad (1)$$

W_0, mass of dry polymer and W_t, swollen polymer mass.

2.2.4. Drug incorporation

The hydrogel samples used in the swelling test were dehydrated in a vacuum oven at 35 °C until constant masses were attained. The weighed dry polymer samples were then incorporated with theophylline by immersing the dry gels in a 800 ppm theophylline aqueous solution at room temperature for 96 h.

2.2.5. Analysis of drug release

Procedures were similar to those outlined in a previous publication (Ng et al., 2002).

2.2.6. Toxicity test

HaCaT cells were seeded in Greiner 96 well tissue culture plates at a density of 1000 cells/100 μl and cultured in Ca^{2+} free Dulbecco's modified Eagle's (DMEM) medium with fetal calf (bovine) serum (FCS) (10%, v/v) in 5% CO_2 at 37 °C. Hydrogel wash solutions were prepared by immersing sterile hydrogel samples in Milli-Q water (20 g/ml) for a period of 14 days. The cells were incubated with sterile hydrogel wash solutions (20 μl) for 48 h as well as with varying concentrations (125, 250, 500, 1000 ppm) of the free form monomers: HMMI, NVP and HEMA. The plates containing the cell cultures were observed under an inverted microscope after the 48 h incubation period and 10 μl of the MTT solution was added to the wells. The plates were then further incubated for 3 h, after which the supernatant was removed and the blue MTT formazan crystals formed were dissolved with 100 μl of the MTT assay solvent (0.1 M HCl in anhydrous isopropanol). The absorbance of the coloured product was measured in a microplate reader (BMG Labtechnologies FLUOstar Optima) at 560 nm with a background absorbance of 650 nm. The increase in the absorbance reading correlated to the cell viability. The untreated cells served as the reference and were assumed as 100% cell viability. The blank medium served as the zero viability reference. The data were expressed as percentage cell viability relative to reference cells. Each treatment was carried out in triplicates.

2.2.6.1. Statistical analysis of MTT assay data. The MTT assay data were expressed as means ± SEM. The cell proliferation data of the hydrogel samples were compared with the free form monomers and also with the experimental control. A one-way analysis of variance (ANOVA) was performed using MINITAB 7.2 statistical software. A P-value of <0.05 was regarded as statistically significant.

2.2.7. FTIR analysis

FTIR spectra of chitosan, Gel A (CS:NVP:HMMI in the ratio of 33.5:62.3:4.2%, w/w) and Gel B (CS:NVP:HEMA in the ratio of 50:25:25%, w/w) were recorded on KBr pellets using a Perkin–Elmer Spectrum One Spectrometer. The software used was Spectrum v3.02, version 3.02.01 from the same company. The sampling range was from 450 to 4000 cm^{-1}. Each sample was averaged over nine scans at 4 cm^{-1} resolution.

3. Results and discussion

3.1. Effect of amount of chitosan to NVP and NVP/HEMA composition in IPN formation

Table 1 gives a summary of the effect of the formulation compositions to the formation of IPNs understudy. All formulations, Formulations (i)–(v) were exposed to a UV dose of 9 kJ. Out of these five formulations, Formulations (i)–(iii) contained CS as well as NVP (donor) and HMMI (acceptor) in varying ratios. The photoinitiator-free photopolymerisation between HMMI and NVP to form hydrogels which were resilient although no crosslinking agent was incorporated in the synthesis has previously been reported (Ng, Swami, & Jönsson, 2004). The mechanism for the initiation of photopolymerisation involving this acceptor/donor pair is clearly detailed in this publication. Formulation (iv) comprised of an acceptor, HEMA and CS.

Formulation (v) consisted of CS as well as an acceptor/donor pair, HEMA/NVP. It was observed that the two formulations, Formulations (iii) and (iv) became viscous, but did not effectively cure over the designated curing time.

Comparing Formulations (i)–(iii), it can be seen that Formulation (iii) having the highest CS with the composition of 62%CS:33.6%NVP:4.0%HMMI resulted in no polymerisation. The contributing factor to this formulation for not polymerising could be insufficient free radicals being formed in the presence of a UV source due to a lower quantity of NVP as compared to CS in the formulation. Formulation (ii) consisted of 48%CS:48%NVP:4.0%HMMI with equal percentage of CS and NVP present in the formulation led to the formation of a polymer which was soluble in water indicating a lack of crosslinking density within the poly(NVP) matrix, as uncrosslinked poly(NVP) is known to be water soluble. Hence, the polymer obtained from Formulation (ii) was not used for further analysis. Only Formulation (i) with the highest NVP composition, comprising of 33.6%CS:62.4%NVP:4.0%HMMI formed IPN which possessed the characteristics of hydrogels with swelling ability.

Formulation (iv), which consisted of HEMA and chitosan, also did not result in the formation of an IPN. This could be due to insufficient formation of free radicals which were essential to initiate polymerisation when HEMA (acceptor) alone in the presence of CS was exposed to a UV source. However, when NVP (donor) was included as in Formulation (v) with the composition of 25%NVP:25%HEMA:50%CS, an IPN which possessed the characteristics of hydrogels with swelling ability resulted. It thus demonstrated that it was essential to have the presence of the acceptor/donor pair, HEMA/NVP under the influence of a UV source in order to produce free radicals to induce polymerisation.

3.2. FTIR spectra

Fig. 1 depicts the FTIR spectra of chitosan, Gels A and B. The characteristic peaks of chitosan were observed at 3479 cm^{-1} (–O–H stretch), 2956 cm^{-1} (–C–H stretch), 1720 cm^{-1} ($–NH_2$ deformation), 1581 cm^{-1} (–N–H bend), 1170 cm^{-1} (bridge-O-stretch) and 1099 cm^{-1} (–C–O–stretch).

Gel A spectrum shows the characteristic peaks of 2375 cm^{-1}; ($–NR_2$ peak), 1683 cm^{-1} (–CON (amide peak)), 1392–1487 cm^{-1} (–C–H bend (saturated)) and 1296 cm^{-1} (–C–O stretch). These peaks indicated the presence of chitosan, and NVP in the polymeric structure.

Gel B spectrum shows characteristic peaks of 2375 cm^{-1}; ($–NR_2$ peak), 1740 cm^{-1} (–COO stretch (ester)), 1683 cm^{-1} (–CON stretch (amide)), 1392–1487 cm^{-1} (–C–H bend (saturated)), 1296 cm^{-1} (–C–O stretch) and 1188 cm^{-1} (–COOR peak). These characteristic peaks show the presence of chitosan, NVP, and HEMA in the structure.

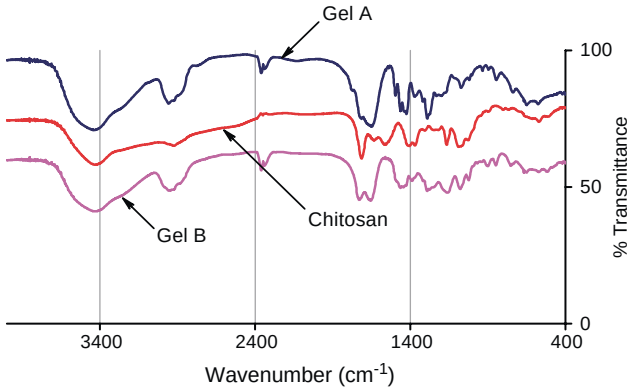

Fig. 1. FTIR spectrum illustrating the characteristics of chitosan and the IPNs, chitosan–NVP (Gel A) and chitosan–NVP–HEMA (Gel B).

3.3. Swelling

Fig. 2 displays the swelling of Gels A and B in Milli-Q water. Gel A composed mainly of chitosan and NVP which were both hydrophilic in nature, displayed high swelling ability with EWC of 93% at the end of 24 h. However, Gel B containing HEMA, a slightly more hydrophobic monomer than NVP had a value of 75% after 48 h.

The relationship, $M_t/M_{\text{infinity}} = Kt^n$ describes the time dependent swelling of a polymer, where K is a constant of proportionality, M_t is the mass of water incorporated at time, t, and M_{infinity} is the mass of water incorporated at infinite time. For Fickian diffusion (diffusion into a cylinder), $n = 0.5$ or M_t/M_{infinity} is directly proportional to the square root of time, and in the case of non-Fickian (anomalous) diffusion, $0.5 < n < 1.0$.

A steep exponential increase in the water uptake by Gel A in neutral pH environment at 37 °C was observed in the first hour indicating the possibility of non-Fickian anomalous transport behaviour. This was confirmed by plotting log M_t/M_{infinity} versus log time (h) based on the swelling test data of Gels A, as shown in Fig. 3. The gradient of such a plot for Gel A had a value of 0.7 confirming that the diffusion of water into Gel A did not adhere to the Fickian model of diffusion into a cylinder. However, in the later stages of the swelling process, Case II diffusion behaviour prevailed which was indicated by a gradual decrease in

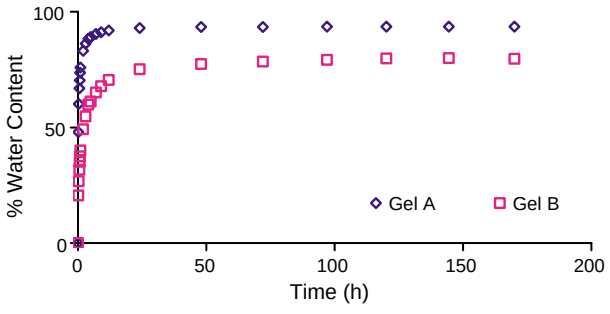

Fig. 2. Equilibrium swelling of Gels A and B in Milli-Q water at 37 °C.

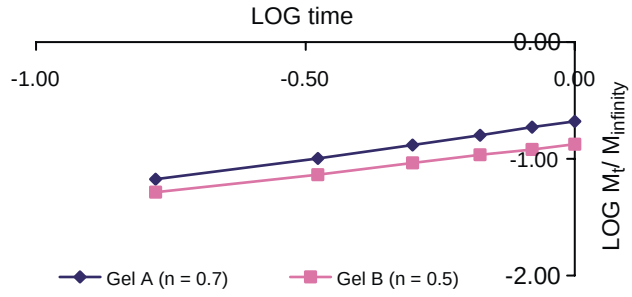

Fig. 3. Plot of log $M_t/M_{infinity}$ versus log time (h) for Gels A and B with swelling experiments performed in Milli-Q water at 37 °C.

the rate of water uptake by the IPNs. The gradual decrease in the rate of water uptake was observed around 7 h of swelling which became constant upon 24 h of constant swelling thus indicating equilibrium water content uptake was achieved.

On the other hand, the gradient for a similar plot for Gel B had a value of 0.5 (Fig. 3) implying that the diffusion of water into the hydrogel adhered to the Fickian model. It thus indicated that the incorporation of HEMA, a more hydrophobic monomer in the formulation resulted in a lower EWC with gradual water diffusion into the polymer core.

3.4. Drug release

As shown in Fig. 4, the drug release experiments conducted on Gels A and B in neutral pH environment using theophylline as the model drug yielded equilibrium drug release (EDR) values of 0.96 and 0.97, respectively, in 24 h. However, within the first hour, Gel A displayed a higher rate of drug release compared to Gel B with fractional drug release of 0.74 and 0.66 for Gels A and B, respectively. Data in Fig. 4 show that the drug release rates increased rapidly in the first 2 h of the experiment after which the release rates gradually slowed down and eventually became constant around 7 h. The quick release of the drug from the carrier in the early stages of the experiment was attributed to the burst effect release. It is a known fact that polymer containing the incorporated solute has to swell to a certain extent before it can release its

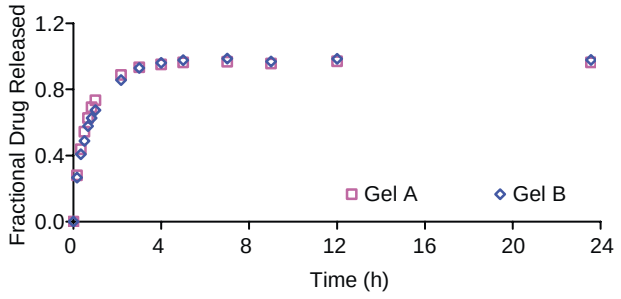

Fig. 4. Plot of the fractional release of theophylline from the IPNs: Gels A and B at 37 °C in neutral pH environment as a function of time.

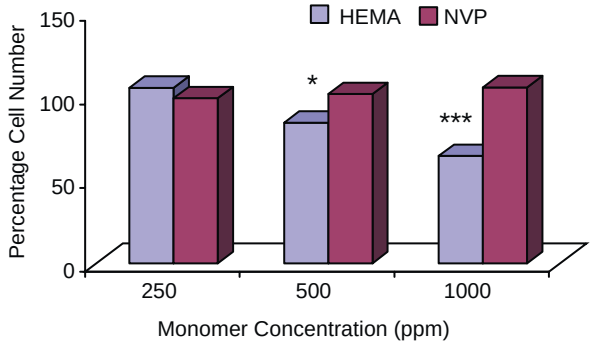

Fig. 5. HaCaT cell growth in HEMA and NVP at 37 °C, *Represents significance where $P<0.05$; ***Represents significance where $P<0.001$.

contents and the resultant effect is the fast release of the solute in a short span of time.

The drug release profile observed in the IPNs could be described as Fickian transport behaviour as the fractional drug released at time t was observed to be directly proportional to the square root of time (graph not shown). Even though Gel B had displayed a relatively low swelling ability as compared to Gel A, a very similar release rate was observed in both Gels A and B. This could be attributed to the relatively low molecular weight of theophylline, which diffuses with ease through the pores of both IPNs at a similar rate.

3.5. Toxicity

The monomers tested for cytotoxicity displayed varied responses to the HaCaT cells. The MTT assay results showed that NVP did not have an adverse effect on cellular viability. Microscopic examination revealed that the cells were firmly attached to the culture dish, indicating the inertness of the monomer. HaCaT cells treated with HEMA (250 ppm) appeared to be viable and had proliferated in this environment. However, when the concentration of HEMA was increased to 500 and 1000 ppm, a toxic effect was observed (Fig. 5).

HMMI on the other hand had adverse effects on HaCaT cells as shown in Fig. 6. The cells treated with HMMI at 125 ppm showed low survival rate (~10%). At higher

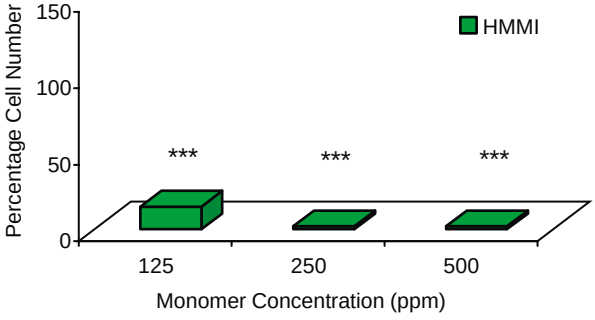

Fig. 6. HaCaT cell growth in HMMI at 37 °C, ***Represents significance where $P<0.001$.

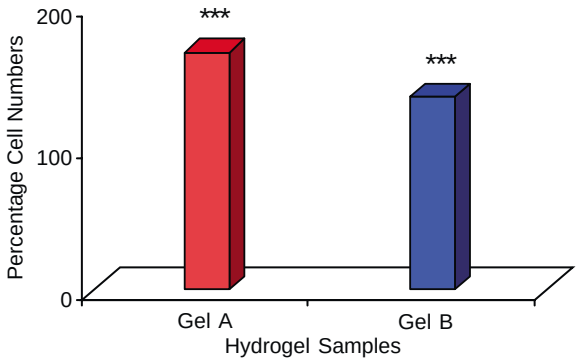

Fig. 7. HaCaT cell growth in Gels A and B at 37 °C, ***Represents significance where $P < 0.001$.

concentrations all the cells died. Microscopic observation indicated that all the cells had detached or had died after being treated with the HMMI at 250 and 500 ppm.

The MTT assay data (Fig. 7) showed significant cell proliferation activity in the presence of hydrogel leachates. Despite apparent toxicity of the monomers, HMMI and HEMA, the hydrogels sustained and significantly stimulated cells growth ($>100\%$). Microscopic observation revealed that the cells were still attached to the culture dish after 48 h of exposure thus indicating that the hydrogels did not have any adverse effect on HaCaT cell viability. The cytotoxicity results thus obtained did satisfy biocompatibility requirements, which state that a suitable biomaterial material should either be inert or have positive response to the host environment. However, increased cell growth may not be desirable at all times and may not be acceptable for some specific bioapplications. Nevertheless, the results of MTT cell proliferation assay on these cells showed that the gels were not toxic to HaCaT cells as indicated by a positive cell growth.

4. Conclusions

IPN hydrogels based on the polysaccharides, chitosan in conjunction with NVP and NVP/HEMA were successfully synthesised through a photoinitiator-free curing technique. However, it was necessary to have NVP and NVP–HEMA in sufficient quantity for efficient curing and adequate crosslinkage in the IPN matrix. The IPN hydrogels thus formed were found to be resilient and functioned competently as drug delivery devices.

Experimental swelling data revealed that the Gel A IPN adhered to non-Fickian anomalous diffusion behaviour in the earlier stages of the experiment followed by Case II diffusion in the later stages. Inclusion of HEMA into the IPN resulted in reducing the swelling activity in the IPN, and adhered to a typical Fickian behaviour.

The drug release experiments revealed that all the IPNs understudy adhered to Fickian transport mechanism in releasing theophylline into the neutral pH environment at 37 °C. The burst effect release of the drug was observed in the initial stages of the release experiment followed by linear release profile. The rate of release of theophylline in all the IPNs under study were found to be similar indicating that theophylline being a low molecular weight drug could diffuse through the IPN hydrogels with ease.

Toxicity tests on these IPN hydrogels involving human keratinocyte, HaCaT showed no evidence of these gels being toxic to human cell culture. Hence, these IPNs can be potential biomaterials for drug delivery applications.

Acknowledgements

The authors are grateful to Dr Narshima Reddy for processing the NMR data.

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