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## Research Article

# IN-VITRO RELEASE STUDY AND ANTIMICROBIAL PROPERTY EVALUATION OF OFLOXACIN LOADED POLY (2-HYDROXYETHYL METHACRYLATE) / POLY (CAPROLACTONE) / POLY (ETHYLENE GLYCOL) HYDROGEL SYSTEM FOR BURN WOUND MANAGEMENT

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## ABSTRACT

Monomer 2-hydroxy ethyl methacrylate containing small amounts of poly(caprolactone) and poly(ethylene glycol) incorporated with an antibiotic ofloxacin was polymerized by photopolymerization technique using 2,4,6 trimethyl benzoyl diphenyl phosphine oxide (TPO) as photoinitiator. Encapsulation efficiency and *in vitro* drug release was studied using UV-visible spectroscopy. Swelling analysis was resorted to compare fluid uptake ability of hydrogel containing the drug with bare polymer. Zone of inhibition assay showed hydrogel containing 1% Ofloxacin to possess strong antimicrobial property Hemolysis assay demonstrated the hydrogel system to be non-hemolytic. Non-cytotoxic character of the hydrogel was confirmed using fibroblast cells. Cell adhesion studies showed non-attachment of fibroblasts to the polymer and improved cell proliferation simultaneously.

**Key words:** Ofloxacin, Encapsulation efficiency, Hydrogel, Antimicrobial

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## INTRODUCTION

Burns are considered as one of the major health problems in the world, which accounts for nearly 2,65,000 deaths per annum. They tend to differ from other types of wounds exhibiting large amounts of non-viable tissue, exudation of tissue fluids, serum and blood, exposed for extended periods and exhibiting colonization of pathogenic bacteria. It is understood that infection of burn wounds is the main cause of increased rate of mortality and morbidity. Bacterial strains associated with burn wounds have been reported to change with time.<sup>1</sup> Both gram-negative and gram-positive bacteria are responsible for burn wound

infection. Topical antimicrobial agents such as silver sulphadiazine are generally used to prevent infection and at the same time, the wound needs to be kept closed to prevent the invasion of microbes.

Hydrogel materials have earlier proved its excellence in wound dressing applications.<sup>2-6</sup> It has the capacity to absorb wound exudates, make the wound clean and impart a moist environment, which facilitates healing. Hydrogel matrix incorporated with an antibiotic/antimicrobial agent is highly recommended for the treatment of an infected burn wound. Some of the reported novel antimicrobial wound dressings are gelatin hydrogel pads containing silver nanoparticles, chitosan

with nano-ZnO, PVA-PVP hydrogel with seaweed extract and silver nanoparticle, poly(2-hydroxy ethyl methacrylate (pHEMA) hydrogel with ciprofloxacin.<sup>7</sup> Ofloxacin (9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid) is a second generation fluoroquinolone antibiotic. It exhibits a broad spectrum of antibiotic property against gram-positive and gram-negative bacteria due to the presence of pyridine carboxylic acid group and is effectively used clinically for treating wound infections.<sup>8-9</sup> Ofloxacin also inhibits the DNA replication by binding with enzymes DNA gyrase and topoisomerase IV which may eventually lead to the death of the microbe. Aqueous solution of ofloxacin shows absorption maxima at 293 nm corresponding to the carbonyl and carboxylic chromophores and the nitrogen of the piperazinyl group. This specific spectral aspect was used for the quantitative analysis during the *in vitro* drug release studies.

In our previous work, we have reported the development of cyto-compatible and non-adherent hydrogel wound dressing material based on pHEMA-PCL-PEG with good swelling and handling property.<sup>10</sup> As infection is the major concern of burn wounds, incorporation of the antimicrobial agent into the above system is envisaged in this study in order to improve the infection control property. Ofloxacin in pHEMA-PCL-PEG hydrogel system is expected to augment the acceptance of pHEMA-PCL-PEG as a wound dressing material due to its antibiotic property. We have discussed here the preparation of this novel hydrogel, its characterization, the *in vitro* drug release profile of antibiotic from the hydrogel and the antimicrobial property demonstrated against gram-negative *E. coli* and gram-positive *S. aureus*.

## MATERIALS AND METHODS

### Materials

2-hydroxyethylmethacrylate [HEMA], 2, 4, 6 Trimethyl Benzoyl Diphenyl Phosphine oxide (TPO) and Polycaprolactone (PCL,  $M_w$  14,000), Ofloxacin (OFX) were all procured from Sigma-Aldrich Chemical Company Inc.(USA). Monomer HEMA was vacuum distilled before use. Polyethylene glycol (PEG, MW200) and formaldehyde were procured from Merck, Germany. Iscove's modified Dulbecco's Minimal Essential Medium – F12, Antibiotic-antimycotic solution, Foetal Bovine Calf Serum, Typsin-EDTA (all cell culture grade) were obtained from Invitrogen, USA. Acridin orange, Ethidium bromide Texas red – phalloidin were obtained from Molecular Probes (USA). All reagents used were of analytical or equivalent grade. Phosphate buffered saline (PBS, pH 7.4) was prepared by dissolving 17.9g of di-sodium hydrogen phosphate, 5.73g of monosodium hydrogen phosphate and 9g of sodium chloride in 1L distilled water. Photopolymerization was carried out using a dental light cure unit containing a halogen bulb (Model 301, Prolite, Caulk/Dentsply, Melfork, DE, USA) of wavelength 380-480 nm was used for photo-curing the monomer.

### Preparation of ofloxacin incorporated hydrogel-using photopolymerization

General methods for loading a drug into a hydrogel system have been reported earlier.<sup>11</sup> In the present work, ofloxacin incorporated hydrogel was prepared using photo-polymerization as per patented procedure.<sup>12</sup> PCL and PEG were added to the HEMA monomer, heated to 70-75°C until a homogenous mix is obtained, antibiotic drug Ofloxacin was added to the homogenous mix followed by the addition of 1wt% TPO with constant stirring. After about 5 min, the solution was poured into a teflon mold (60 mm x 10 mm x 1 mm) and exposed to a dental light source for 3-5 minutes. After photo curing was complete, the mold containing the cured polymer was kept in distilled water for maximum of 2hrs to facilitate easy removal of the cured polymer. Table 1 shows details of the hydrogel systems prepared.

Table 1: Sample code and composition of polymeric hydrogels

Sample Code	Wt %			Drug (Wt%)
	HEMA	PCL	PEG	Ofloxacin
Hdrg-0.25	85	10	5	0.25
Hdrg-0.5	85	10	5	0.5
Hdrg-0.75	85	10	5	0.75
Hdrg-1.0	85	10	5	1.0
HI-3	85	10	5	0

### Swelling studies

Swelling study is important as fluid absorbing capacity is one of the important properties of hydrogel which provides a moisture environment over the wound bed. Pre-weighed lyophilised samples of Hdrg1 having dimensions 6mm x 1mm were immersed in de-ionized water at 37°C for 24 hours. After removing the adherent water, the swollen samples were again weighed at specific intervals of time using an analytical balance

(Model CP224S, Sartorius, Germany) having a sensitivity of 0.1mg. The swelling percentage of the samples were determined according to the following equation

$$\text{Percentage swelling, } S\% = \left\{ \frac{[W_s - W_o]}{W_o} \right\} \times 100 \dots \dots \dots (1)$$

where  $W_s$  and  $W_o$  are the masses of the swollen and dry samples respectively

### Surface Wettability Studies

Surface wettability of the dried hydrogel systems was determined via the sessile drop method using a video-assisted contact angle measuring device (Data Physics OCA15 plus, Germany) and imaging software (SCA20 software, Germany). Within 10 seconds of introduction of the DI/W droplets, the contact angle formed between the sessile droplets and the matrix was measured. The contact angle is expressed as an average value of four independent measurements taken at different sites on each matrix.

### Encapsulation Efficiency

About 4mg of lyophilized and powdered Hdr1 was incubated with 100 ml of PBS for complete swelling. The mixture was transferred to a 50 ml centrifuging tube and agitated using a sonicator (Bandelin Sonorex Digitec, Germany) for 10 minutes. After centrifugation (2000 rpm, 10 minutes), the supernatant solution was analyzed using UV-Visible spectroscopy (Shimadzu, Japan) at a wavelength of 293 nm. Drug encapsulation efficiency was calculated using the following equation:

#### Drug Encapsulation Efficiency =

$$\frac{\text{Amount of drug present in the matrix}}{\text{Theoretical amount of drug load}} \times 100 \dots\dots\dots(2)$$

### In vitro release profile of OFX drug

Total immersion method was used to study the *in vitro* release of ofloxacin drug from cured and swollen hydrogel systems. After removal from the Teflon mold, hydrogel discs of 6mm diameter and 1mm thickness were punched out and incubated with 2ml PBS buffer (pH 7.4) in a shaking incubator at 37°C and 50 rpm for 24 hr. After every 24 hr, UV absorbance was measured and equal volume of buffer replaced with the fresh buffer at each measurement time points. Readings were taken upto 120 hr at 24 hr intervals. A calibration curve of ofloxacin was earlier carried out in the range of quantity, within which a linear relationship between the concentration and absorbance was realized. From the calibration curve, the eluted drug concentration was determined. The experiments were carried out in triplicates and reported as average values.

### Antimicrobial activity

Antimicrobial activity assessment of the hydrogel systems Hdr1.0 (Hydrogel systems with highest drug loading) and HI-3 (bare without drug) was carried out using Agar diffusion method based on ISO 20645:2004(E) procedure against gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. Inhibition zone around the drug-incorporated hydrogel was compared after 24 h incubation at 37°C, with a standard antibiotic disc as positive control and pHEMA-PCL-PEG hydrogel without drug as negative control (HI3). A minimum of 3 samples were tested in each case.

### Hemolysis Assay

Hemocompatibility testing of hydrogel systems was carried out broadly on the basis of ISO 10993-4:2002(E) selection of tests for interaction of materials with blood. The test is mainly aimed at finding the extent of hemolysis caused by the sample. Blood from a healthy human volunteer was collected and the anticoagulant CPD-A was added as required. The samples kept in PBS were taken out and placed in polystyrene plates. Two ml of blood was added to each sample, one ml blood was taken immediately for initial analysis and 1ml blood was incubated with the samples for 30 min under agitation at  $70 \pm 5$  rpm using an Environ shaker thermostated at  $35 \pm 2^\circ\text{C}$ . Four empty polystyrene culture dishes were exposed with blood as reference. The total hemoglobin in the whole blood samples was measured using an automatic hematology analyzer (Sysmex-K 4500). The free hemoglobin liberated into the plasma after exposure to materials was measured using a diode array spectrophotometer (HP 8453 Hewlett-Packard GmbH, Germany) and the hemolysis percentage was calculated using the following formula:

$$\% \text{ Hemolysis} = (\text{Free Hb} / \text{Total Hb}) \times 100 \dots\dots\dots (3)$$

### Cytotoxicity by direct contact assay

An *in vitro* cytotoxicity test using the direct contact method was performed using fibroblast cells (Discarded foreskin). Hdr1 and control HI-3 samples were sterilized using ethylene oxide. The sterilized samples were then immersed in culture medium containing serum for 30 min prior to testing and placed on the cells (40000 cells/cm<sup>2</sup>) and medium was replaced with fresh medium. After incubation at  $37 \pm 1^\circ\text{C}$  for 48 h, the cell monolayer was imaged using optical microscope and Leica Application Suit software (Leica, DMIRB, Germany) at a magnification of 10X.

### Cell viability study/ Live dead assay

Cell viability was analyzed by live-dead assay using acridine orange and ethidium bromide. Briefly, the fibroblast cells were cultured for 48h in direct contact with the hydrogel matrix at 37°C in a humidified CO<sub>2</sub> incubator in DMEM: HAM F12 medium containing 10% FBS. After 48h, the hydrogel was removed and the cells were washed with sterile PBS (thrice). Cells were incubated with acridine orange (1:100 dilution or 0.1 µg / ml) and ethidium bromide (1:100 dilution 0.1 µg / ml) in PBS at room temperature for 20 min. Cells were washed with PBS thrice at 10-min intervals and images were captured using a fluorescent microscope and Leica Application Suit software (Leica, DMIRB, Germany) at a magnification of 10X.

### Cell Adhesion study

Hydrogel samples were seeded with a cell density of 70000 cells /cm<sup>2</sup> and allowed to attach for 72hours and then fixed with 3.7% formaldehyde for 20 minutes and then permeabilized with 0.2% triton X 100 (Molecular probes USA). Texas Red-conjugated antibodies (1:500) against actin cytoskeleton were added and kept at 37°C for 30 min, washed thoroughly using PBS and observed through fluorescent microscope (Leica DMIRB, Germany) at a magnification of 20X.

## RESULTS

### Fabrication of the Ofloxacin-incorporated hydrogel matrix

Photopolymerization at 380-480 nm of the drug containing monomer system was found to yield a hydrogel in which the drug was incorporated fully. Figure 1 shows the ofloxacin-incorporated swollen hydrogel sheet after the polymerization.

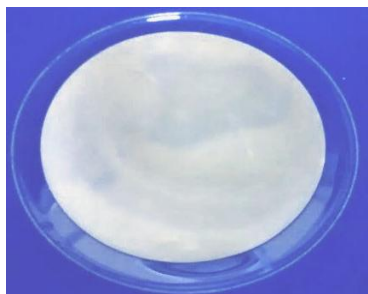


Figure 1: Ofloxacin-incorporated swollen hydrogel matrix (Hdrg)

### Characterization of Ofloxacin-incorporated hydrogel matrix

#### Swelling analysis

To analyze the effect of incorporated drug on the fluid uptake ability of hydrogel system, swelling analysis was carried out in distilled water. The results of the swelling analysis of hydrogel systems are depicted in Figure 2. Comparative evaluation of swelling capacity indicated that fluid uptake ability of Hdrg1 ( $40 \pm 2\%$ ) was found to be significantly lower than that of HI-3 ( $64 \pm 2.8\%$ ).

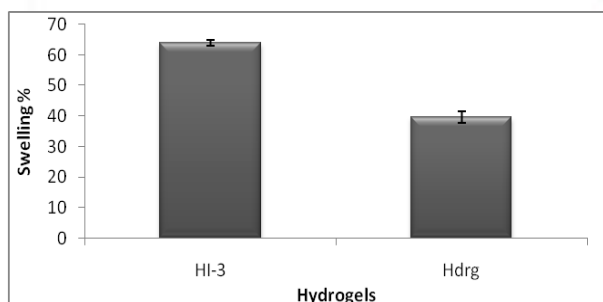


Figure 2: Swelling analysis of hydrogel systems

### Surface Wettability Study

The observed value of the contact angle of Hdrg1 was found to be  $61 \pm 2^\circ$ . It was observed that addition of drug increased the surface hydrophobicity of the matrix compared to bare matrix HI-3 ( $54 \pm 1^\circ$ ).

### Drug Encapsulation Efficiency

Evaluation of drug encapsulation efficiency is an important aspect, which provides a quantitative estimation of the actual drug within the hydrogel after formulation. UV spectroscopy technique was used for this study. Figure 3 depicts the UV absorption spectrum of Ofloxacin. Percentage encapsulation efficiency of ofloxacin in the hydrogel system was found to be nearly 60%.

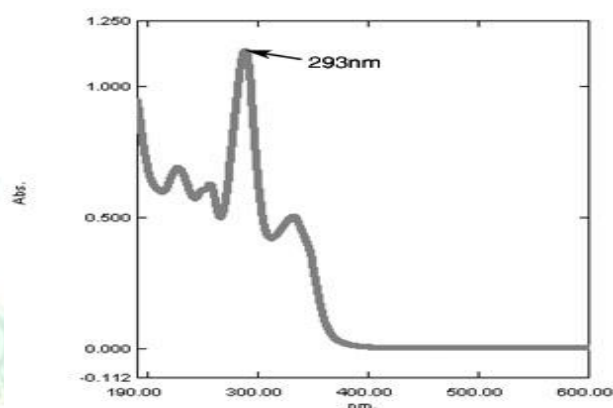


Figure 3: UV Absorbance spectrum of Ofloxacin

### *In vitro* release study of ofloxacin

#### Effect of medium

Drug release study conducted in distilled water and PBS at pH=7.4 demonstrated a faster drug release in PBS compared to water. The complete release of the drug from the matrix was completed in nearly 120h in distilled water, whereas in PBS, sustained release was observed for 120 h after the initial burst release in the first 24 hrs (Figures 4 A and B).

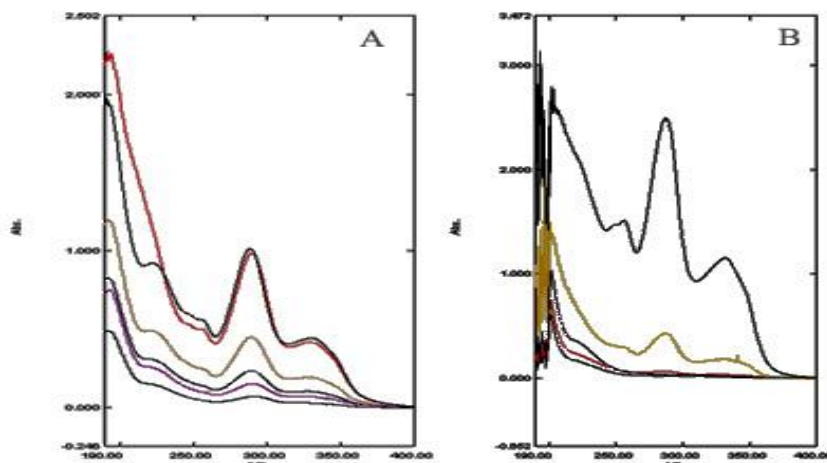


Figure 4: Release study of ofloxacin A) water B) PBS

### Effect of drug loading

Figure 5 displays the release profile of ofloxacin from hydrogels at different amounts of drug loading. The data shows that the hydrogel systems containing the highest amount of the drug exhibited a faster release rate than that of the systems with less drug loading. Hdr1 showed a faster rate of drug release while Hdr0.25 displayed a slower rate of drug release.

### Antimicrobial property evaluation

The antimicrobial property of the hydrogel matrix with different drug content was evaluated against gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria using the agar disc diffusion method. Figures 6 display zone of inhibition assay of Hdr1 against *Staphylococcus aureus* and *Escherichia coli*. The zone of inhibition of Hdr1 was found to be similar with that of the control disc (Table 2).

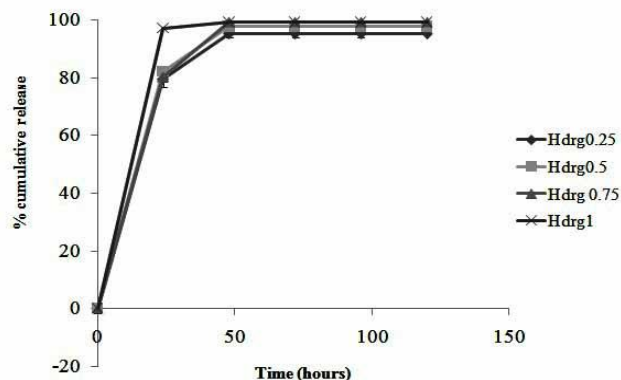


Figure 5: Cumulative release of drug from hydrogel systems with different drug loadings (n≥3)

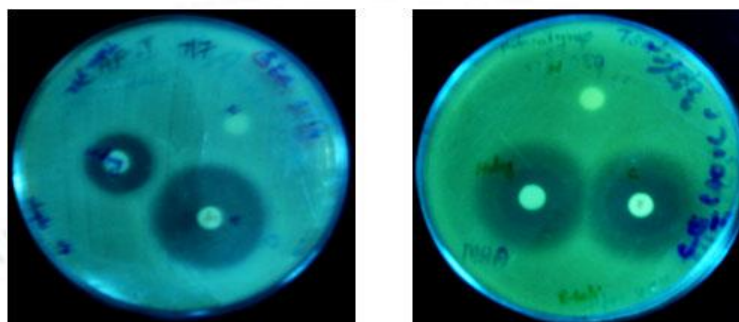


Figure 6: Representative patterns of zone of inhibition assay of Hdr1 against (A) *Staphylococcus aureus* (B) *Escherichia coli*. (n≥3)

Table 2: Zone of inhibition of HI-3(negative control), Hdr1 and Antibiotic drug (positive control) against *S.aureus* and *E.Coli*

Samples	Zone of inhibition	
	<i>S.Aureus</i> ATCC 25923	<i>E.Coli</i> ATCC 25922
	Unit 1	Unit 1
HI-3	Nil	Nil
Hdr1	12mm	20mm
Control	33±0.5mm	20 ±1.5mm

### Hemolysis Assay

Hydrogel system without drug was found to be non-hemolytic. To study the effect of drug incorporation on

hemolytic property of hydrogel system, % hemolysis assay was conducted and was found to be  $1.56 \pm 0.3\%$ .

### In vitro cytotoxic evaluation

#### Cytotoxicity by direct contact assay

Cytotoxic evaluation of the hydrogel system containing higher drug concentration (Hdr1) was performed using direct contact assay. Figures 7 A, B and C show the microscopic images of control fibroblasts, fibroblasts incubated with HI-3 and Hdr1 respectively. Incubation of the fibroblasts with Hdr1 for 48h did not have any undesirable effect on the cells as evidenced by retention of spindle-shape morphology as well as good spreading (Figure 7C).

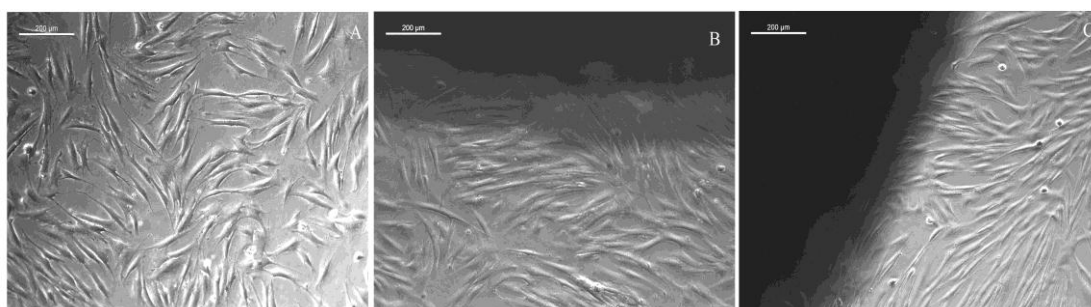


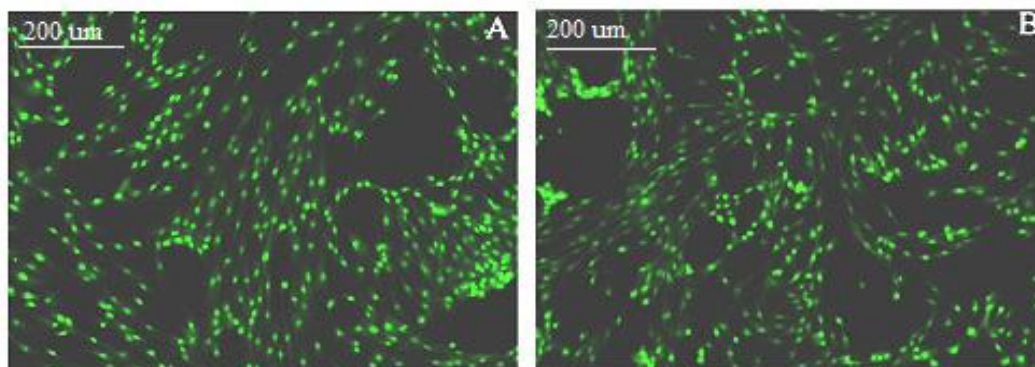
Figure 7: Microscopic image of fibroblasts (A) control cell (B) contact with HI-3(C) With Hdr1 (n≥3)

### Cell viability

Acridine orange and ethidium bromide staining on fibroblast cells after incubation with HdrG1 for 48 hours was conducted to evaluate the cell viability. Figures 8 A and B displays the fluorescent microscopic images of control fibroblasts and fibroblasts incubated with HdrG1. Cell counting was carried out using Image J software and nearly 100% of the cells were found to be viable after incubation with HdrG1 for 48h.

### Cell adhesion study

Non-adherence to fibroblast cell is found to be an important quality of the wound dressing material. Figures 9 G, H and I depict the microscopic images of actin staining on control fibroblast cells, HdrG1 hydrogel and fibroblast cells in contact with HdrG1 respectively. Cell adhesion study was conducted using actin staining exhibited non - adhesion of the fibroblasts to HdrG1 (Figure 9 H) while the fibroblast cells grown underneath the hydrogel HdrG1 exhibited good spreading and spindle shaped morphology (Figure 9 I).



**Figure 8: Live-dead assay using Acridine Orange and Ethidium Bromide on fibroblast cells, incubation at 37°C for 48h (A) Control fibroblasts (B) HdrG1 (n≥3)**



**Figure 9: Microscopic images of actin staining on (G) Control fibroblast cells (H) HdrG hydrogel (I) Fibroblast cells in contact with HdrG1 (n≥3)**

## DISCUSSION

Various methods have been reported for preparing drug-incorporated hydrogel matrices for biomedical applications. Song *et al* showed that effective incorporation of a drug can be achieved by incorporation of a drug in the monomer mixture along with the initiator and other additives and then allowing it to polymerize.<sup>11</sup> In the present study, we have resorted to photo-polymerization technique successfully to prepare HdrG (Figure 1) hydrogel by addition of a drug in the monomer HEMA containing PCL and PEG and a photo-initiator. The role of PCL and PEG in the hydrogel has been explained in detail in our earlier published data.<sup>10</sup> Ofloxacin has been found to be effective earlier in controlling skin structure infection caused by gram-negative and gram-positive aerobic bacteria.<sup>13</sup> It is recommended that burn wound infections can be

effectively reduced by the application of wound dressings incorporated with antibacterial agents rather than by applying topical ointments. The drug delivery system was developed for the purpose of bringing, taking up, retaining, and releasing the drug in right dose at the site.<sup>14,15</sup> Various methods such as dipping or pasting have been adopted for incorporating the antibiotic agent silver sulphadiazine in pHEMA systems to develop antimicrobial burn wound dressings. However, long-term diffusion efficacy and fast elimination of the drug made these techniques ineffective.<sup>16</sup> Thermal methods for impregnation and immobilization of antimicrobial agents onto pHEMA membranes have been reported earlier.<sup>17-19</sup> Tsou *et al* used a UV irradiation technique to prepare poly (2-hydroxy ethyl methacrylate) wound dressing systems incorporated with ciprofloxacin.<sup>7</sup>

Effect of incorporating the drug on swelling property compared to bare hydrogels can be observed in Figure 2. It is seen that incorporating ofloxacin tends to decrease the swelling rate of the hydrogel significantly and improve the hydrophobic character. The contact angle study also substantiates this observation. These results imply that the addition of the drug to the hydrogel system had an influence on its surface as well as on its bulk properties.

The drug encapsulation study indicates that nearly 60% drug is entrapped within the matrix effectively using the photo polymerization method. Figures 4A & B exhibit the burst release of the drug from the matrix within the first 24 hours in PBS compared to that in water. Nearly 85% of the drug was found to be released within 24 hours. Ions present in the PBS may have facilitated the burst release of drug from the matrix. Different drug loading on to the hydrogel matrix was done to evaluate its effect on drug release and antimicrobial effect. Figure 5 displays the release profile of drug from hydrogel at different drug loadings. Release data shows that formulations containing the highest amount of the drug displayed faster and higher release rates than those of the formulations with a lower amount of drug loading. The release rate becomes quite slower at the lower amount of drug in the matrix due to the availability of more free void space for the passage of the drug molecules.

Due to its broad spectrum of antibacterial activity, ofloxacin has been clinically proven earlier to be effective in controlling various infections such as skin and soft tissue infection, respiratory tract infections, urinary tract infections, etc. Figures 6A and B show the clear inhibitory zones exhibited by the HdrG1 against Gram-positive and Gram-negative bacteria. As can be seen from the size of the inhibitory zones (Table 2), similar antibacterial activity is exhibited by both sample and control against the *E.coli* strain. Figure 6A indicates that the antibacterial activity of HdrG1 against *S.aureus* is less compared to control. The antibacterial effect of HdrG1 is found to be more pronounced against Gram-negative bacteria than Gram-positive bacteria. Reports have proven that ofloxacin exhibits greater inhibitory action against gram-negative bacteria compared to gram-positive bacteria.<sup>20</sup> Sato *et al* reported that ofloxacin is the most effective fluoroquinolone against staphylococci.<sup>21</sup> The antibacterial activity of the fluoroquinolone has been attributed to the inhibition of the A subunit of the enzyme DNA gyrase, which prevents the super coiling of bacterial DNA. Furthermore, ofloxacin may have an additional inhibitory effect on the B subunits of the enzyme.<sup>20</sup>

Hemolytic property evaluation of HdrG was carried out as per standard ISO 10993-4:2002 (E) and the results

indicate that incorporation of ofloxacin in the matrix did not induce any hemolysis and the % hemolysis ( $1.56 \pm 0.3\%$ ) was within the permissible level of 5%. *In vitro* cytocompatibility evaluation using the direct contact test indicated that neither the released drug nor the drug within the matrix produced any unfavorable effect on the fibroblast growth. Figure 7C shows the microscopic image of well spread out, healthy spindle-shaped fibroblast cells in case of HdrG1 comparable to fibroblast behavior found in HI-3 (Figure 7B). Nearly 100% of the fibroblast cells were found to be viable after incubation with HdrG1 for 48 hours (Figure 8B). The cell viability assay substantiated the *in vitro* cytocompatibility property of HdrG1. Cells underneath the hydrogel system were also found to maintain their spindle-shaped morphology with a well spread out nature (Figure 9I). These results validate the earlier non-cytotoxic property of the HdrG1. Studies of actin staining indicated that the introduction of drug in the matrix does not produce any change in the non-cell adhesion property of the hydrogel system (Figure 9H).

## CONCLUSION

Photopolymerization using a light source at 380-480nm was found effective in incorporating ofloxacin drug in a hydrogel system containing poly(HEMA) along with PCL and PEG. Incorporation of Ofloxacin at 1% level in the HdrG1 hydrogel system was found to exhibit strong antimicrobial property as evidenced by zone of inhibition assay. Cytotoxic assessment by indirect contact method of HdrG1 revealed the hydrogel non-cytotoxic to fibroblasts. Fibroblasts were found not to adhere to the hydrogel while exhibiting improved cell proliferation. The hydrogel also exhibited good swelling, non-hemolytic character and optimum hydrophobic/hydrophilic balance for potential application as wound dressing.

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## Conflict of Interest

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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