Chapter 5

CMT Polysaccharide based ternary hydrogel for biomedical application

The results of this chapter has been compiled to Manuscript (under preparation)

5.1 Introduction

In the previous chapters-3, the role of hydrophilic monomer in functionality modification of TKP has been investigated. In chapter-4, the incorporation of hydrophobic pendant group and the consequent functionality transformation on modified TKP have been investigated. The impact of functionality modification of polysaccharides on cell adhesion and growth of different cells has been studied. Further investigation includes the effect of these changes (functionality modification) on polysaccharide based biomaterials for drug encapsulation efficiency and in vitro release kinetics of hydrophilic drug from these biomaterials. However the quest for assessment of incorporating both these hydrophilic and hydrophobic pendant groups on polysaccharide backbone and consequent impact of biophysical behaviour of resultant biomaterials has lead investigation included in this chapter. The detailed synthesis and characterisation of ternary polysaccharide based hydrogel have been discussed in this chapter. Further this chapter also includes study of changes in biophysical properties and their effect on potential biomedical applications.

5.2 Results

5.2.1 Synthesis

The ternary hydrogel was synthesized using two different monomers 2-HEMA and AA by radical polymerisation using benzoyl peroxide as the initiator. Further the ternary hydrogel were synthesised by varying four different parameters; mole composition of AA and HEMA, initiator concentration with respect to HEMA and time of reaction. All these parameters were varied at three levels as discussed in details in Chapter-2. The incorporation of AA and HEMA had taken place to different extent which had no correlation with monomer composition. However the extent of grafting of particular monomer were mainly affected by reactivity of that particular monomer and time of reaction. The AA incorporation
had taken place to higher extent owing its higher reactivity as compared to HEMA. This was further corroborated as despite of higher mole ratio of HEMA taken in case of S3 the incorporation had taken to lesser extent. The incorporation of AA had taken place more in S3 despite of it low concentration (Table 2.1). These were concluded from the observations made in swelling study and grafting yield as discussed in succeeding sections.

5.2.1.1 Grafting yield

The grafting yield was gravimetrically determined with formula mentioned elsewhere (Chapter 2). Fig 5.1 gives a comparative plot of grafting yield (%) of ternary hydrogels. The grafting yield was found to be highest for S3 and S9 hydrogel which eventually have higher extent of AA incorporation in the CMT backbone.

![Grafting yield](image)

**Figure 5.1** Gravimetrically determined grafting yield (%) of ternary hydrogels with different mole compositions of AA and HEMA.

5.2.2 FTIR Spectroscopic analysis

The FTIR spectral analysis gives important information about the functionality and microstructural modification on polymer backbone. The FTIR spectra of pure CMT, CMT-g-HEMA (1:10) and CMT grafted with both AA and HEMA were displayed in Figure 5.2. The prominent peaks for each matrix has been summarised in Table 5.1. The characteristic peaks observed are OH, C=O, C-O, COO-, CH₂, & C-O-C.

A broad peak around 3150–3460 cm⁻¹ is observed corresponding to O-H stretching frequency for all the polysaccharide based matrices. For pure CMT there are two observed peaks at 3600 and 3160 cm⁻¹. This may be attributed to alcoholic and carboxylic peak respectively for pure CMT. However these two peaks subsumed to single broad peak for all the hydrogels to around 3350 to 3460 cm⁻¹. This shift takes place due to incorporation of COOH group and
thus gives a combined broad peak in the above region. This shift was observed upto maximum of 3460 cm\(^{-1}\) in case of S3 sample. This trend may be attributed to highest incorporation (or grafting) of AA in S3 ternary hydrogel. Appearance of a peak at 1010-1092 cm\(^{-1}\) corresponds to C-O-C linkage. This peak was observed in case of all the hydrogels except pure CMT where a very minor peak was observed at 1094 cm\(^{-1}\). The pendant group links to the polysaccharide backbone through C-O-C linkage formed at C-3 of glucan unit as that position was thermodynamically favourable for grafting of AA or/and HEMA. The variation of C-O-C peak in wide frequency range may be attributed to the type of pendant group (HEMA or AA) that grafts at C-3 position and also affected by the number of pendant group forming the ether linkage. The higher extent of HEMA grafting gives a higher frequency of C-O-C linkage but higher extent of AA grafting leads to ether (C-O-C) linkage in lower frequency region. The carboxylate (COO\(^{-}\)) peak at 1720 cm\(^{-1}\) was observed in case CMT:HEMA mole ratio of 1:10. However this peak was either absent or a minor off- soot can be observed in some of the hydrogels (S3, S5, S8, and S9). This can be attributed to lower extent of grafting of HEMA on to the polysaccharide backbone. The peak at 1650 to 1660 cm\(^{-1}\) can be observed for all the hydrogels that can be attributed to C=O stretching frequency and lower than normal carbonyl frequency at 1700 cm\(^{-1}\) due to resonance that decreases the full double character of C=O linkage. From different peak position it can be inferred that the grafting of AA monomer has taken place with higher extent as compared to HEMA across the ternary hydrogels of different composition AA and HEMA. This trend may be attributed to proximity of reactants with similar polarity facilitating easier grafting reaction. It can be believed that the relatively hydrophilic monomer acrylic acid and hydrophilic polymeric backbone of CMT polysaccharide comes to proximity which allows for easier grafting of acrylic acid monomer. However the monomer HEMA due its relative hydrophobicity probably gets preferentially less access to polysaccharide backbone for covalent interaction to take place and thus mechanistically hindered HEMA from grafting on to the polymer backbone. Moreover, the monomer HEMA have been grafted to lesser extent through covalent interaction and to some extent the HEMA incorporation within polymer matrices might have taken place through physical entanglement through weak Van der Waals interaction. Thus it can be inferred that the complex polymer network structure resulted wherein polysaccharide mostly grafted chemically with acrylic acid and to lesser extent with hydrophobic HEMA by both physically entanglement and grafting through covalent interaction.
**Fig 5.2** FTIR spectra of different polysaccharide based matrix. Transmittance vs. Wave number (in cm$^{-1}$)

**Table 5.1** The characteristic peak of different hydrogels summarised below:

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Prominent peaks in cm$^{-1}$</th>
<th>Remark (Significant peaks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMT Pure</td>
<td>3600, 3160, 2929, 2884, 1657, 1592, 1423, 1309, 1150, 1094.</td>
<td>O-H peak at 3149 – 3420 cm$^{-1}$ with significantly lower frequency for CMT Pure.</td>
</tr>
<tr>
<td>CMT-g-HEMA (1:10)</td>
<td>3348, 2929, 2856, 1720, 1632, 1430, 1304, 1168, 1076.</td>
<td>C=O peak 1720 cm$^{-1}$, C-O-C peak at 1018-1094 cm$^{-1}$, COO$^-$ asymmetric stretching at 1660 cm$^{-1}$, COO$^-$ symmetric stretching at 1420 cm$^{-1}$, O=C-O stretching frequency at around 1160 cm$^{-1}$.</td>
</tr>
<tr>
<td>S1</td>
<td>3400, 2930, 1652, 1420, 1154, 1040.</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>3390, 2932, 1650, 1440, 1159, 1082.</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>3460, 2929, 2861, 1653, 1439, 1326, 1160.</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>3420, 2930, 1650, 1422, 1165, 1016.</td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td>3383, 2929, 2862, 1650, 1430, 1157, 1021.</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>3410, 2930, 1650, 1420, 1048.</td>
<td></td>
</tr>
<tr>
<td>S7</td>
<td>3410, 2927, 1648, 1424, 1157, 1042.</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>3430, 2927, 1654, 1430, 1158, 1048.</td>
<td></td>
</tr>
<tr>
<td>S9</td>
<td>3423, 2929, 1650, 1430, 1165, 1018.</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3 Swelling study

The swelling behaviour gives vital information about transformation in microstructural pattern of the polymer due to chemical modification through grafting of monomers on to polysaccharide backbone. There is a remarkable increase in hydro-swelling behaviour for S3 sample followed by S9, S1 and S4 respectively. Although there is an irregular pattern in hydro-swelling behaviour for different hydrogels with respect to mole composition of AA and HEMA, however in general, duration of reaction (time of reaction) played a key role for incorporation of hydrophilicity. The increased hydrophilicity may be attributed to incorporation of more acrylic acid through grafting by covalent interaction with polysaccharide backbone. Only few of the hydrogels (S1, S3, S4, S7, S8 and S9) have demonstrated significant amount of swelling (Fig. 5.3). The other hydrogels (S2, S5 and S6) were extremely hydrophobic and have almost insignificant or no swelling ability as observed under similar conditions.

![Graph](image.png)

**Figure 5.3 a)** Swelling study showing percent swelling vs. time (in minutes) for different hydrogels. The S3 sample has highest hydrophilicity amongst ternary hydrogels and S8 sample least hydrophilicity. **b)** Enlarged view of highlighted area showing swelling variation of different hydrogel within first 400 mins.

It was inferred from swelling study that the higher hydrophilicity was due to higher extent of grafting of AA on CMT backbone. For S1 hydrogel the higher grafting may be attributed to high initiator concentration and reaction time. Because the synthesis of S1 sample involves lower mole ratio of AA and moderate concentration of HEMA, however the hydrophilicity of the ternary hydrogel has increased markedly which may be attributed to incorporation of acrylic acid into polymer backbone. In case of S9 hydrogel, preferential swelling of AA was due to higher mole composition of AA (higher reactivity than HEMA).
The incorporation of HEMA may be further inferred from FTIR spectral analysis, as HEMA incorporation has given a minor peak at 1712 cm\(^{-1}\) which was subsumed with carboxylate peak at 1647 cm\(^{-1}\). This might have enhanced the relative hydrophobicity with respect to S3 hydrogel and thus substantially increasing the hydrophobicity of the S9 hydrogel (Fig. 5.2). This anomaly may have occurred because the lower extent of HEMA incorporation by covalent interaction and which didn’t affect appreciably the pore size and accommodate quite a large amount of water molecules.

5.2.4 XRD analysis

The X-Ray Diffraction analysis was carried out to assess the role of micro-structural transformation on crystallinity that has resulted due to grafting of HEMA on CMT polysaccharide backbone. The polysaccharide shows semi-crystalline micro-structural arrangement under ordinary conditions. However grafting results in micro-structural changes at the molecular level that create crystalline domains. The crystalline domain acts as reinforcing grids, provides mechanical strength to polymeric network and improve the material performance over a wide range of temperature. It seems from XRD peaks, the relative intensity varies with hydrophilicity and extent of grafting (Fig. 5.4). The lower peak intensity for S3 hydrogel may be ascribed to lesser number of crystalline domains present within hydrogel micro-structure partly due to its hydrophilicity owing to the AA grafting. It can be ascertain that the creation of crystalline domain was associated with hydrophobic part of alkyl chain of pendant group and assisted by hydrophobic association leading to crystalline domains. Fig.5.4b represents the extent of crystallinity in terms of percent crystallinity measured by comparing area covered under intensity peak of crystalline and amorphous domain as mention elsewhere in chapter-2. The Fig.5.4 depicts crystallinity in general decreases with increasing hydrophilicity of the hydrogels. It can be observed that the S3 hydrogel with highest hydrophilicity has lowest intensity peak corresponds to lower extent crystalline domains. Thus the most hydrophilic S3 hydrogel, has lowest intensity due to lower number of crystalline domains as evidenced from lowest percent crystallinity (38%). In contrast the S1 and S9 which were relatively less hydrophilic or alternatively can be said to have higher hydrophobicity which aids in creation of higher number of crystalline domains leading to higher crystallinity.
Figure 5.4 a) XRD pattern of polysaccharide based matrices with compositional variation in dual grafted monomers. The ternary hydrogel S1, S3 and S9 represents grafted hydrogel with different mole ratio of AA and HEMA. The XRD pattern shows variable intensity for different samples owing to different extent of crystalline domain in polysaccharide based matrixes. b) Crystallinity (%) of different matrices.

5.2.5 DLS analysis

The surface charge of the hydrogel was assessed by measuring zeta potential value. The negative potential shows the poly-electrolytic nature of the hydrogel throughout the compositional variation of AA and HEMA incorporation in ternary hydrogel and also includes the similar surface characteristics for pure polysaccharide (CMT). The negative potential is due to the presence of carboxylate ion both from CMT and AA. The variation in negative charge is representative of the variation in extent of incorporation of pendant group into polysaccharide backbone. There is a significant decrease in surface potential of different ternary hydrogels in comparison to pure polysaccharide (CMT). This may attributed to decrease in charge contributing groups along with an increase in the pendant groups. The pendant groups contributed to the non-ionic alkyl groups on the surface of the hydrogel matrix and there was decrease in exposed charge contributing groups. Additionally the HEMA incorporation also decreases the surface charge due to absence of charge contributing groups unlike AA incorporation which partly contributes charge due to the presence anionic carboxylate ions. More over the long chain hydrophobic alkyl groups of the pendant groups sequester the charged groups that contributed to the surface charge resulting in decreased surface potential for the hydrogels. The higher surface potential is essential for particle stability and in turn long duration coherence in the material property of the hydrogel matrices. Further the surface charge also plays a key role in cell adhesion and hence surface
charge assessment provides important clue for material suitability in bioapplications. Amongst the hydrogels, S1, S3 and S9 possessed with modest zeta potential value desirable for higher stability. Further these three hydrogels also have shown higher hydrophilic character than the rest of other hydrogels which eventually would increase their material efficacy for cell adhesion and growth on their material surfaces.

![Zeta potential value for different polysaccharide based matrixes.](image)

**Figure 5.5** Zeta potential value for the different polysaccharide based matrixes. There was significant change in surface charge for different hydrogel with varying degree of grafting of monomer.

5.2.6 Morphological analysis

The surface topographic feature and micro architecture was analysed from SEM micrographic images. The most hydrophilic ternary hydrogel was taken for SEM analysis and compared with pure polysaccharide (CMT) (Fig.5.6). In the fig it was observed that the S3 hydrogel has micropores however similar micro pore were not observed with pure polysaccharide (CMT). The micro-porosity was created probably due to a network structure formed in the ternary hydrogel on incorporation of pendant groups. The porous architecture is a hallmark of materials suitability for different bio-applications such as drug delivery devices and scaffolds for tissue engineering applications. It enhances drug encapsulation and retention ability and also controls the release of drugs. Further the pore architecture supports tissue ingrowth and mass transport, desirable for scaffolding materials.


**Figure 5.6** SEM images showing pattern of pore structure. (a-b) Pure CMT, and (c-d) S-3 ternary hydrogel. The material samples were hydro-swelled and lyophilized for SEM micrographic analysis. The S3 hydrogel shows microporosity within the pore structures however this is not observed with pure CMT polysaccharide. The microporosity created may be attributed to grafting of the monomers to the polysaccharide backbone.

### 5.2.7 Biocompatibility assay

#### 5.2.7.1 Cell viability with Saos-2 cells

Biocompatibility is a prerequisite condition for any material for application in living systems. Therefore it is essential to check the biocompatibility of ternary hydrogels of different compositions to find a suitable composition for specific bioapplication. The hydrogels with different compositional variation of dual monomer was assessed by MTT assay with osteoblast Saos-2 cells (**Fig.5.7**). The MTT assay involves a control in which cells were grown in absence of any hydrogel shows 100% cell viability. Further the cell viability was observed below 100% on pure CMT polysaccharide. However there was a significant increase in viability (above 100%) for S1, S3 and S9 hydrogels. Notably these three ternary hydrogels were possessed with higher hydrophilicity due to increased content of AA. The lower viability in cases of S2 & S4 may be attributed to the hydrophobic content due to HEMA despite of biocompatible nature of HEMA.
Figure 5.7 MTT Assay for different hydrogels with Soas-2 cell showing the cell viability as a function of material.

5.2.7.2 Adhesion and growth of RAW 264.7

The study of biocompatibility next involves a comparative analysis of growth pattern of pre-osteoclast cells (RAW 264.7) on different material surfaces. Fig.5.8 shows the fluorescent microscopic images of cell growth on different polysaccharide based matrices. It can be observed that the cells survive and grow on all the material surfaces including in control, where it grows without the material support. It can be inferred from these observations that the polysaccharide based material surfaces are biocompatible with bone precursor RAW 264.7 cells.

Figure 5.8 Growth of RAW 264.7 cells on ternary hydrogels: RAW 264.7 cells are seeded on material coated cover slip and incubated for 24 hours at 37°C and 5% CO₂. Cells are fixed by 4% PFA solution and nucleus of cells is stained by DAPI and images are taken by fluorescence microscope.
5.2.7.3 Adhesion and growth of Saos-2 cells

Subsequently, the biocompatibility was assessed further by growth analysis of osteoblast-like Saos-2 cells on different ternary hydrogel surfaces. Fig.5.9 depicts the confocal microscopic images of Saos-2 cells shows a comparative growth pattern of cells. The actin filament and nucleus can be observed in all the samples under investigation including control wherein the cells were grown without any material support i.e. only on glass surface. There is no significant variation of growth pattern of the cells except for S3 where a ‘sealing zone’ pattern can be observed. These are the well defined focal adhesion points at the bottom surface of the cells which were growing on the hydrogels and suggests about the adherent nature of Saos-2 cells on S3 hydrogel. This can be attributed to the specific characteristics of osteoblast-like Saos-2 cells. It was found that Saos2 cells effectively adhere and have grown on this surface. Staining of actin filaments with Phalloidin (Red) suggest that Saos2 cells forms a “sealing-zone like structure” at the lower surface (Fig.5.9). Such a “sealing-zone”, i.e. a ring-like structure is a hallmark of specific signaling process where morphological changes in the podosomes occurs [Luxenburg et al. 2007; Nakamura et al. 1999; Anderegg et al 2011; Geblinger et al 2012; Geblinger et al. 2010]. It can be demonstrated here that the most hydrophilic hydrogel (S3) offers better material property for growth and adhesion of osteoblast-like Saos-2 cells.
Figure 5.9 Shown are the confocal images of Saos-2 cells grown on CMT:AA:HEMA ternary hydrogel surface. Cells were grown for 60 hours of on this surface before fixing. Cells were stained for polymerized F-actin fibre by Alexa-594 labelled phalloidin (Red) and DNA by DAPI (blue). An enlarged view of the culture is shown in the lower panel. a) Saos-2 cells grown on glass surface are shown, b) S1 hydrogel c) S3 hydrogel d) S4 hydrogel. No significant differences were observed when Saos2 cells were grown on glass surface or on hydrogel surfaces.

5.2.8 In vitro drug dissolution kinetics

Controlled in vitro drug release efficacy of the ternary hydrogel was assessed by USP-Type-II apparatus under physiological pH (7.2) and temperature (37°C). The hydrogel was tested for releasing ability of the drug paracetamol (a hydrophilic composition). The observation from chapter-3 & 4 in which the polysaccharide based matrix with both hydrophilic and hydrophobic pendant groups have shown different release efficacy. To further proceed with the investigation about-role of the either hydrophobic or hydrophilic character on release efficacy, the present study involves the ternary hydrogel incorporating both the hydrophilic and hydrophobic pendant groups on the polysaccharide backbone.

Further the encapsulation efficiency of the different hydrogels was compared in Fig.5.10a. It was observed that the encapsulation efficiency was highest (54%) for the moderately hydrophilic hydrogel (S9). The hydrophilic hydrogel S1, S3 & S9 shows higher encapsulation efficiency (49%, 49% and 54% respectively). However the relatively less hydrophilic hydrogels, S2 and S4 has lower encapsulation efficiency with 44% and 46% respectively. The controlled release efficacy from ternary hydrogel with compositional variation of two different grafted monomers on CMT backbone was also tested. Fig.5.10b depicts the in vitro dissolution kinetics of paracetamol from different matrices. It can be observed from Fig.
5.10b that the most hydrophilic hydrogel S3 shows faster release kinetics of paracetamol. However the hydrogel S9 with moderate hydrophilicity has the most controlled release kinetics of drug molecule. The hydrophobic hydrogel S2 also shows comparatively faster release kinetics than the moderately hydrophilic hydrogels S1 and S4. This demonstrates that the hydrogel with moderate hydrophilicity shows better result in terms of controlled release kinetics for hydrophilic paracetamol. The S9 hydrogel with higher encapsulation efficiency shows the most controlled release pattern amongst the ternary hydrogels. This is attributed to the encapsulation of drug molecule taken place mostly by absorption. However S3 hydrogel despite higher encapsulation releases the drug molecule at a faster rate which can be ascribed to the encapsulation of drug molecule mostly through adsorption.

![Figure 5.10](image.png)

**Figure 5.10** a) Encapsulation efficiency of different ternary hydrogels, b) *In vitro* release kinetics of paracetamol from different hydrogel matrices.

5.3 Conclusion

A ternary hydrogel with different hydrophobic/hydrophilic characteristics was successfully prepared from the dual grafting of AA and HEMA on CMT backbone polymer. The results demonstrate that the hydrogel had promising biophysical properties such as enhanced adhesion, biocompatibility, cyto-compatibility and controlled release kinetics for hydrophilic drug subject to predominance of hydrophobic/hydrophilic character.