Synthesis and characterization of Carboxy methyl tamarind polysaccharide based hydrogel (CMT-g-HEMA) for biomedical application

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4.1 Introduction

In the preceding chapter the role of hydrophilic monomer AA has been explored and studied extensively. It has been observed that with increasing hydrophilic pendant group there is increase in hydrophilicity of tamarind kernel polysaccharide along with increasing mechanical properties with the network structure resulted by grafted hydrogel. Further there is the enhanced cell adhesion for different cell lines apart from satisfying the matrix requirements for controlled drug delivery devices. This chapter deals with the investigation of role of hydrophobic pendant group (2-HEMA) in tamarind polysaccharide backbone on their biomedical application such as cell adhesion and efficacy of drug encapsulation and controlled release behavior. Thus it would be interesting to investigate the role of hydrophobic pendant group on physico-chemical modification on hydrogel matrix and effect on biomedical application thereon. However the base polymer chosen in this study was carboxy methyl tamarind polysaccharide (CMT) as the polysaccharide backbone instead of tamarind polysaccharide. TKP has certain inferior material property such as lower water solubility and easier degradation which limits its suitability for bioapplication. The carboxy methylated counterpart of TKP, namely CMT has been reported to have enhanced water solubility and stability against degradability and therefore increases its material suitability for diverse potential biomedical application.

4.2 Results

4.2.1 Synthesis

For the synthesis of hydrogels, the reaction was carried out by using different mole ratios of CMT and HEMA in the presence of Benzoyl Peroxide as initiator. In
order to observe the changes in the appearance of the hydrogels, photographic images of the final products were considered (Fig. 4.1). The optical properties indicate the relative changes in grafting as the respective color of the hydrogels range from near-white translucent to creamy white color as the CMT and HEMA ratio changes from 1:0.5 to 1:10. As the ratio changes further, the respective colors became darker again (from ratio 1:20 to 1:50). At a ratio of 1:50, the two components reveal two different layers indicating a phase separation. We attribute this trend in color to the increment in grafting with increased mole ratio for HEMA. This increment reaches its maxima when CMT: HEMA mole ratio became 1:10. Further increment in the HEMA ratio results in less grafting and also results in phase separation (when CMT and HEMA was used at 1:30 as well as 1:50 mole ratios). As these hydrogels were not grafted properly, these materials with less grafting properties were not characterized further.

**Figure 4.1** Photographic images showing the reaction mixtures with different mole ratios of CMT: HEMA as indicated respectively.

### 4.2.1.1 Grafting yields

The grafting yields were determined gravimetrically for different hydrogels. Fig. 4.2 shows a comparative plot for percent grafting yield of different hydrogels. The percent grafting yield in general increased with increase in HEMA mole composition and significantly higher yield was observed for hydrogel with 1:10 mole composition, however the yield decreased with further increase in HEMA mole composition (1:20). This may be probably due to the high concentration of HEMA (at 1:20 mole ratio and higher), the homo-polymerization of HEMA has predominantly taken place that had significantly decreased the grafting of HEMA on to the polymer backbone.
4.2.1.2 SDS-PAGE

The purity of synthesized hydrogels was assessed from SDS-PAGE analysis as purity of hydrogel matrices are quintessential for their bioapplications. It can be observed from the Fig.4.3 that the polysaccharide CMT after grafted with HEMA and subsequent purification had no protein band and hence was free from impurities. This was not observed with pure CMT which was also undergone similar purification process of solvent washing with acetone. This was alike TKP-g-AA hydrogel where grafting has enabled separation of copolymer from protein impurities and for the reason discussed in Chapter 3.

Figure 4.2 Gravimetrically determined grafting yield (%) of the hydrogels with different mole composition of CMT and HEMA.

Figure 4.3 SDS-PAGE image showing protein free hydrogel matrices were formed by grafting of monomer in contrast to pure CMT which retains its protein content post purification.
The hydrogels were characterized by different spectral and physical analysis such as UV-visible, FTIR, swelling study, DLS, XRD, SEM, etc. The details are illustrated in following sub-sections.

4.2.2 UV spectroscopic analysis

These materials were characterized by UV visible spectra and FTIR spectra. Fig.4.4 compares the variation in absorbance of hydrogels with incorporation of HEMA on to the CMT backbone. Figure shows, there is an increase in absorbance with increasing mole composition of HEMA with respect to pure CMT. This increase in absorbance indicates about the increasing extent of grafting of HEMA to the CMT (Fig.4.4). Acquiring UV spectra for 1:10 ratio of CMT: HEMA was not possible as at this ratio it formed suspension in water.

![Figure 4.4 UV-visible spectra of hydrogels with different mole ratio of CMT to HEMA.](image)

4.2.3 FTIR spectroscopic analysis

The hydrogels were analyzed by FTIR spectra, collected in the range 500-4000 cm\(^{-1}\) (Fig.4.5). All the hydrogels of different mole ratio of CMT: HEMA have characteristic broad peak for hydroxyl group at 3600 cm\(^{-1}\). The bands at 2925 and 2850 cm\(^{-1}\) are due to asymmetric and symmetric C-H stretching vibrations [Sen et al. 2009]. The peak at 1132 cm\(^{-1}\) is due to C-O stretching. Further, the peak around 1620 and 1414 cm\(^{-1}\) corresponds to asymmetric and symmetric stretches of COO\(^{-}\) for pure CMT [Sen et al. 2009]. There is a shift in asymmetric stretching peak to 1565 cm\(^{-1}\) for COO\(^{-}\) after grafting takes place for different hydrogels. However the intense peak at 1620 cm\(^{-1}\) reappears for 1:20 mole ratio of CMT: HEMA which can be attributed to the decrease in grafting percentage. The shift of CO peak from normal carbonyl (1735 cm\(^{-1}\)) peak to
around 1570 cm\(^{-1}\) corresponds to a change in bond arrangement into one and half bond linkages in carboxylate (COO\(^{-}\)) [Ibrahim et al. 2005].

![Image of FTIR spectra](image)

**Figure 4.5** FTIR spectra of hydrogels of different CMT: HEMA compositions. a. CMT: HEMA (1:20), b. CMT: HEMA (1:10), c. CMT: HEMA (1:20), d. Pure CMT.

The extent of grafting was tested by using FT-IR spectroscopy (Fig.4.6). These results also reveal increased grafting properties with the increment in HEMA mole ratio with respect to CMT. In accordance with the optical properties, the IR spectroscopic analysis also indicates that the highest grafting occurs for CMT: HEMA at a mole ratio of 1:10, beyond which it declines again. The IR spectroscopic analysis elucidates insertion of HEMA on CMT backbone. The grafting yield was calculated from IR analysis and gravimetric trend (Sec 4.2.1.1). These results also reflect that CMT and HEMA produce the best grafted product at a mole ratio of 1:10 (Fig.4.6).

![Image of area profile](image)

**Figure 4.6** The grafting profile as calculated from FTIR for hydrogels with increasing HEMA mole ratio to CMT.
4.2.4 Swelling study

In order to characterize these materials further, swelling study was performed. Hydro-swelling kinetics reflects relative increment in hydrophobic character of hydrogels. The swelling study depicts that there is decrease in quantity of water present as the HEMA content increases in the hydrogels (Fig. 4.7). This suggests that the hydrophobic character of hydrogel is increasing with HEMA content. However, the increment in hydrophobicity reaches its maxima with 1:10 ratio of CMT: HEMA composition and then decreases as shown in the case of 1:20 composition (Fig. 4.7).

Figure 4.7 Swelling kinetics of hydrogels with different mole ratios of CMT to HEMA.

4.2.5 DLS analysis

The surface charge of material plays a key role in adhesion, growth and the survival of cell on the material surface and therefore it is essential to measure the surface charge of the material. The surface charge of hydrogel matrices was assessed from the zeta potential measurement at pH 7.0. Fig. 4.8 shows a comparative surface charge for different hydrogels. Fig. 4.5 depicts that there is no regular trend of surface charge variation with respect to HEMA incorporation on polymer back bone. It can be observed that the zeta potential decreases for CMT-g-HEMA at mole ratio of 1:1 with comparison to Pure CMT and thereafter increases with incorporation of HEMA and highest negative potential was attained at 1:5 mole ratios. This trend can be correlated to exposed charged carboxylate ion with HEMA incorporation on polysaccharide backbone. With further increase in HEMA results in intra-molecular H-bonding between carboxylate ion and hydroxyl group owing to their favorable proximity which
decreases the exposed carboxylate ion. This in result decreases zeta potential of the hydrogel with higher grafting of pendant group HEMA.

Figure 4.8 Zeta potential value of CMT-g-HEMA hydrogel with different mole ratio of their respective composition. The composition (1:0) represents pure CMT.

4.2.6 XRD analysis

The X-Ray Diffraction analysis was carried out with different polysaccharide based hydrogel to assess the effect of incorporation of hydrophobic pendant group on microstructural/molecular reorganization leading to crystallinity. Fig.4.9 shows the comparative crystallinity pattern in different polysaccharide based hydrogel molecular reorganization pattern that resulted due to grafting of HEMA on CMT backbone. There is incremental change in intensity at 18° with mole ratio of HEMA with respect to CMT. This can inferred as increase in crystalline domain with corresponding increase in HEMA pendant group. The lowest intensity for pure CMT may be attributed least molecular reorganization in absence of hydrophobic pendant group. As there is incorporation of HEMA takes place, the molecular reorganization induced by hydrophobic pendant group is prominent resulting in increase of crystalline domains.
4.2.6 Thermo Gravimetric Analysis (TGA)

All copolymers have shown identical degradation profile in four steps alike pure CMT (Fig. 4.10). However, with the extent of grafting, the tendencies towards degradation in stage 1 have increased as well. Increase of the grafting in copolymers (CMT: HEMA with 1:5 & 1:10 mole composition), a sharp degradation was observed just above 400°C. It was probably due to the degradation of grafted extensions of the polymer chains, which were present in higher numbers in these hydrogels because of higher HEMA incorporation on CMT backbone. However degradation of these hydrogels at such a high temperature has no practical significance for their efficacy in bioapplications.
4.2.7 Morphological study

The surface morphology and pore architecture provide important clues whether a material is suitable for bioapplications such as drug delivery and in particular tissue scaffolds. Because right surface morphology and pore architectures are determinant factors for cell adhesion and mass transport required for cell growth and proliferation. **Fig.4.11** shows SEM images for polysaccharide based matrix. The SEM images of pure CMT (**Fig.4.11a-b**) shows there is undulated surface morphology without any vascularised pore architecture. However the hydrogel CMT-g-HEMA at mole ratio (1:10) shows well vascularised pore architecture along with undulated surface morphology (**Fig.4.11c-d**)  

![SEM images showing surface morphology (a-b) Pure CMT & (c-d) CMT-g-HEMA (1:10). The morphology shows that there is increased vascularised microstructure for HEMA grafted CMT hydrogel as compared to pure CMT. (scale bar 100 µm).](image)

4.2.8 Biocompatibility

4.2.8.1 Cell viability with Saos-2 cells

The MTT assay was carried out to assess the viability of Saos-2 cells on CMT-g-HEMA hydrogel surface. **Fig.4.12** gives a comparative plot of viability (%) of Saos-2 cells on different hydrogel surfaces. The figure depicts the higher viability of Saos-2 cells on hydrogel surfaces as compared to control (i.e. without the support of any material surface). The cell viability was not affected significantly with extent of grafting, however highest viability was observed for hydrogel with 1:10 mole
composition of CMT and HEMA. Thus this confirms the absence of any cytotoxicity of the material and biocompatibility towards the Saos-2 cells.

**Figure 4.12** Cell viability of CMT-g-HEMA hydrogel with different mole composition of HEMA.

### 4.2.8.2 Adhesion and growth of pre-osteoclast RAW 264.7 and osteoblasts like Saos-2 cells

The biocompatibility assessment of the hydrogel is necessary to find its potential for application in biomedical field. The study in this section involves the assessment of biocompatibility of CMT: HEMA (1:10) hydrogel. For that purpose, the hydrogel sample were coated as a small spots on the glass cover slips and tested for cell growth on these surfaces, especially on the hydrogels. Different cell lines were used for assessment of compatibility with hydrogel surface, RAW 264.7, Saos-2 and HUVEC etc. which represent osteoclast-precursor cells, osteoblasts like cells and sensitive primary human cells respectively [Hase et al. 2008; Islam et al. 2007; Dalbeth et al. 2008; Pederson et al. 2008; Vääränen. et al 2000].

The effect of hydrogel (CMT: HEMA with 1:10 mole composition) on the growth of RAW 264.7 cell line was studied. It was observed that number of RAW 264.7 cells growing on the hydrogel was much more when compared with the number of cells that are growing on the glass surface only (Fig.4.13). This uneven distribution with maximum cellular density on the hydrogel coated area indicates a specific and positive effect of CMT: HEMA (1:10) hydrogel on the adhesion of RAW 264.7 cells. For that purpose, the distribution of actin cytoskeleton was analyzed by staining the cells with Alexa-488 labeled phalloidin. The focal adhesion points at the hydrogel surface were analyzed. It was observed that the presence of multiple well defined focal adhesion
points at the bottom surface of the cells which are growing on the hydrogels and suggests for a more adherent nature of the RAW 264.7 cells on this surface (Fig.4.13b-c). The dense growth of cells was observed for a long duration, i.e. even after 4th day of plating, indicating that this hydrogel is fully compatible with this cell for prolonged culture.

**Figure 4.13** CMT: HEMA (1:10) hydrogel is biocompatible for bone precursor cells. a). Cells were grown on hydrogels (borders of such hydrogel spot is indicated by green line), fixed and the nucleus of the attached cells were stained with DAPI. Cells effectively grow on the hydrogel surface. b). Shown are the confocal images of cells grown on CMT:HEMA surface. Cells were grown for 24 hours on this surface before fixing. Cells were stained for polymerized F-actin fibre by Alexa-594 labelled phalloidin (Red) and DNA by DAPI (blue). c). Enlarged 3D merged images of the culture are shown. The top view (left), bottom surface view (middle) and ortho-representation (right) indicate the filopodial structures, focal adhesion points and the surface attachments in details.

As RAW 264.7 cells reveals crowded growth specifically on the hydrogel surface 24 hours after plating. Then it was explored if these cells directly adhere on the hydrogel specifically at the early time points or migrate later on due to any chemo attractant activity exerted by CMT:HEMA (1:10) hydrogel. In this technique, the RAW 264.7 cells were labeled with a fluorescent dye and live cell imaging was performed to explore the cell adhesion properties (Fig.4.14). Herein the cell adhesion was visualized on the hydrogel surface as well as on glass cover slips. The faint green auto fluorescence property of the dried hydrogel was used to demarcate the exact border of the CMT: HEMA (1:10) hydrogel spot on the glass cover slip. On plating a RAW 264.7 cell suspension on the cover slips, it was noted that more number of cells specifically adheres on the hydrogel surface and remains attached throughout the imaging time.
Cells were found to attach on the hydrogel surface earlier than on the glass cover slips. **Fig.4.14** depicts the cell adhesion at different time point and it can be observed that cells adhere more on the hydrogel surfaces. This demonstrated that RAW 264.7 cells prefers to adhere directly on CMT: HEMA (1:10) hydrogel.

The hydrogels were tested for biocompatibility with osteogenic Saos-2 cell. **Fig.4.15** shows the adhesion and growth of osteoblasts like Saos-2 cells on glass surface and hydrogel (CMT: HEMA at 1:10) surfaces. The characteristic feature of Saos-2 cells which forms a sealing zone structure was observed on hydrogel surfaces; however this feature was not observed on glass surfaces. This demonstrates that the hydrogel surface was biocompatible with osteogenic cells and the material can have potential application in bone tissue engineering.

Next the study involved the compatibility assessment of this surface for potential application in human. **Fig.4.16** depicts the adhesion and growth of human specific cells on hydrogel surfaces and compares with that of glass surfaces. For that purpose, very sensitive primary cells from human origin, namely Human Umbilical Vein Endothelial cells (HUVEC) were used and tested if HUVEC cells can grow on the CMT:HEMA (1:10) surface. It was observed that the endothelial cells have grown normally on this surface (**Fig.4.16**). No difference was observed in the cellular morphology in the HUVEC grown on glass coverslips or on CMT: HEMA (1:10) surface. This demonstrated that the hydrogel surface bears no cytotoxicity for future application in humans.
Figure 4.14: Specific adhesion of cells on the hydrogel surface. Shown are the live cell experiments demonstrating the adhesion of cells on the hydrogel surface (indicated by the white border line). Cells were stained with a fluorescently active dye and the cell suspension was added to the hydrogel surface for settling of cells. Green, blue and white arrows indicate the cells that adhere directly to the hydrogel surface, to the hydrogel border and to the glass surface respectively.

![Fluorescence images of cells on hydrogel surface](image)

**Figure 4.15** Adhesion and growth of osteogenic cells. Shown are the representative fluorescence images of cells stained for Phalloidin (red) and DAPI (blue). Osteoblasts like Saos-2 cells were grown on only glass (upper panel) and on the CMT-HEMA surface (lower panel) are shown.

![Fluorescence images of osteoblasts](image)
Figure 4.16 Biocompatibility of Human Umbilical Vein Endothelial Cells on CMT: HEMA (1:10) surface. a) Shown are the phase contrast images of HUVEC cells grown for 60 hours on CMT: HEMA surface (i-ii) in lower magnification (i) and higher magnification (ii). Similarly cells grown on glass surface are also shown (iii). The border of the hydrogel surface with cells is shown in inset (i). b) Confocal images of HUVEC cells grown on CMT: HEMA 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. c) Confocal images of HUVEC cells grown on glass surface are shown. No differences were observed when HUVEC cells were grown on glass surface or on CMT: HEMA (1:10) surface.
4.2.9 *In vitro* drug dissolution study

The drug dissolution study was carried out to assess the material efficacy for controlled release of the drug. Paracetamol was taken as the standard drug for the study. The encapsulation efficiency of the drug molecule by CMT-g-HEMA hydrogel with different mole composition was assessed. Fig.4.17a shows comparative plot for encapsulation efficiency by different hydrogel matrices. The hydrogel with highest extent of grafting (1:10 mole composition) had shown lowest encapsulation efficiency (51%). The efficiency had shown a decreasing trend with the extent of grafting. The drug release pattern from hydrogel based matrices also varied with extent of grafting. Fig.4.17b demonstrates that the matrices either with no grafting or lesser extent of grafting (Pure CMT, CMT: HEMA with 1:1 & 1:20 mole composition) releases drug at very faster rate as compared to hydrogel with better grafted hydrogels. Thus hydrogels (with 1:5 and 1:10 mole composition of CMT: HEMA) which has better grafting extent had shown a slower release of drug molecules. Amongst hydrogels (CMT-g-HEMA with 1:10 mole composition) had shown the most controlled release of drug molecules. Further, the hydrogel with lowest encapsulation shows a slowest release pattern. This attribute may be related to drug encapsulation mechanism and the encapsulation of drug molecule was believed to be taken place mostly through absorption into the hydrogel matrix. This was owing to the relatively hydrophobic nature of hydrogel which was more pronounced with extent of grafting (1:10 mole composition) that led to the slower absorption of drug molecule within the hydrogel matrix wherein the drug molecules replaces the bound water from the matrix. The Fig.4.17a depicts there is burst release pattern of drug molecules during initial period (within the first 20 mins) of time after which a sustained release pattern was observed. However there was a transition from burst release pattern to controlled release pattern for hydrogels with higher extent of grafting (1:5 and 1:10 mole ratio) within the same initial period of time. This may be attributed to the better network structure of hydrogel with extent of grafting. The above mentioned encapsulation and release mechanism was further corroborated as most networked hydrogel (CMT: HEMA with 1:10 mole composition) has the highest extent of grafting and also had shown a slowest release pattern (Fig.4.17b). It can be ascertained that the release of drug molecules from the matrices were assisted by reverse hydrodynamic pressure exerted by water molecules which replaces the drug molecules from the matrices in dissolution media.
Figure 4.17 a) Encapsulation efficiencies of different matrices, b) Dissolution kinetic study showing controlled release of hydrophilic drug from different hydrogel matrices. The most controlled release pattern was observed from hydrogel (CMT: HEMA 1:10).

4.3 Micro-structural analysis from FTIR

Based on the FTIR spectral analysis, a probable mechanism was proposed (Scheme 1). Previously Sen and Pal have showed that grafting of acrylamide on to CMT backbone takes place through O-atom (Sen et al. 2009). This study suggests a similar micro-architecture in the CMT-HEMA-based hydrogel. The IR peak at 1120 cm$^{-1}$ in grafted hydrogel indicates the grafting has taken place through the O-atom forming C-O-C linkage. The intensity of this peak increases with increment in grafting percentage corresponding to the increase in HEMA content. The absence of carbonyl peak around 1700 cm$^{-1}$ suggests most probably the graft co-polymerization has taken place without ring opening during or after grafting of 2-HEMA onto the CMT backbone. The prominence of peak intensity at 2925 cm$^{-1}$ may be attributed to the increase in grafted polymerized HEMA.
Proposed mechanism:

\[ \text{Scheme 4.1 Proposed mechanism for grafting of 2-HEMA on CMT polysaccharide} \]

4.4 Conclusion

The Carboxy Methyl Tamarind polysaccharide was successfully modified by incorporating hydrophobic pendant group on to CMT backbone. This was achieved by very simple synthetic methodology namely graft copolymerization reaction using radical initiator as catalyst. The methodology involves a very simple purification process i.e. repeated solvent washing with acetone yields pure grafted product. The simplistic post synthesis process adds further advantage for future industrial application. The synthesized hydrogel shows good biocompatibility with different cells with improved mechanical strength due to the network structure that increases the utility of material for different potential biomedical application. Further, the hydrogel with optimal hydrophilic-hydrophobic balance shows controlled released ability for hydrophilic drugs.