Chapter 7

Summary & Conclusion

The present study in this thesis involves investigation of the material efficacy of tamarind kernel polysaccharide and Carboxy Methylated Tamarind kernel polysaccharide (CMT) for different biomedical applications in particular for controlled drug delivery and bone tissue engineering. The study involves the functionality modification of TKP and CMT and the consequential effect on their material efficacy for biomedical applications. The results have been discussed in following succeeding sections.

Chapter 1 involves an introduction to polysaccharides, their application in different fields, and their potential application in biomedical field either in unmodified or modified and in derivatised form. Further this chapter also discusses the scope and objective of the study enshrined in this thesis. This chapter also deals with the structural chemical information about the polysaccharide, namely TKP chosen for the study.

Chapter 2 discusses on the details of materials used, sample preparation techniques involved in the present investigation and details of the methods used for analytical characterization and application study.

The study in Chapter 3 involves synthesis of a polysaccharide-hydrogel based biomaterial sourced from a very low cost natural polysaccharide, TKP. The TKP based hydrogel was assessed with different physico-chemical analysis for making a suitable hydrogel based biomaterial. It was found from different physico-chemical characterisation that the hydrogel has enhanced property for biological applications. The detailed techniques involved in synthesis and characterisation of this biomaterial has been discussed in chapter 3. The hydrogel TKP-g-AA was found to have higher swelling ability with increased mole ratio of AA incorporation into the polymer backbone. Further the hydrogel had shown enhanced growth of different cells such as Neura2a, F11, RAW 267.4, Saos-2, HUVEC and MC3T3 E1 etc. and supports for better adhesion on the matrices. This was attributed to the surface functionality and micro-architectural transformation within polymer network due to monomer incorporation satisfying structural requirements that allows for mass transport and cell
ingrowth. The detailed surface properties and micro-architecture has been discussed in chapter 3.

It was shown that the hydrogel derived from this polysaccharide (TKP) offers a suitable surface that can potentially be exploited for bone tissue engineering. This work demonstrates that TKP-AA hydrogel is biocompatible and effectively promotes adhesion and growth of different cells such as RAW 264.7, Saos-2, MC3T3 E1, F11, Neuro2a and HUVEC as well as primary osteoblasts and osteoclasts from rodents. The physico-chemical properties and biocompatibility to cells having non-osteogenic and/or osteogenic properties suggest that this material can be potentially used for bone tissue engineering. Previously it was reported that different functional groups such as carboxyl groups, carboxymethyl groups, polyacrylamide groups, phosphate groups etc. alters material hydrophilicity [Rana et al. 2011; Giovanna et al. 2008]. The polysaccharides on cross-linking through these functional groups alter functionality of polymers and are often critical for biomedical application. Herein, the TKP was modified by grafting with acrylic acid (AA). The -OH functionality of polysaccharides was used for further modifications through grafting of poly-AA as pendant group.

This work demonstrates that TKP-AA has good porosity, undulating surface morphology, suitable surface charge, better water retention capacity and compatible with osteogenic as well as non-osteogenic cells. Both primary and secondary cells were used in this study and demonstrated that cells with osteogenic properties can proliferate easily on this surface for a long duration without any symptom of cytotoxicity and form dense clusters (till 100 hours as observed in this study). This indicates that the TKP-AA surface offers positive adhesion and focal signalling events relevant for adhesion and further growth. The fast adhesion and further growth as well as clustering of osteoclasts and osteoblasts on this surface are most likely due to the compatible properties of the cell adhesion molecules, specific receptors, ion channels and intrinsic affinity of this cells for complex carbohydrate molecules. In agreement with this, it was demonstrated that the cells adhere well on this surface and their filopodial structures are well spread. The focal adhesion points are distinct. This observation is in accordance with several reports suggesting that integrin and focal adhesion-mediated signaling events play key roles in bone cell adhesion, chemotaxis, growth, differentiation and maturation [Carvalho et al. 2003; Cheng et al. 2001; Damsky 1999; Kim et al. 2007; Nakayamada et al. 2003; Salasznyk et al. 2007]. The early events of cell adhesion and further growth also suggests that the hydrogel matrix (TKP-g-AA at 1:5 mole
composition) offers suitable alternative material surface which is better than few other surfaces prepared before where cell adhesion is either poor and takes long time or the surface exerts cytotoxicity [Lai et al. 2010].

The TKP-g-AA hydrogels were analysed for in vitro dissolution efficacy of Paracetamol. The results have demonstrated that the hydrogel (CMT: HEMA with 1:5 mole composition) has the highest encapsulation efficiency and the most controlled release pattern for drug molecules. Moreover the hydrogel with this composition has moderate hydrophilicity as well as the extent of grafting. This pattern can be attributed to the altered hydrophilicity due to grafting of AA on TKP backbone at 1:5 mole composition provides optimal network structure that can hold maximum drug molecule within the matrix. Thus it can be concluded that this matrix provides best material efficacy at moderate hydrophilicity as evident from the fact that, apart from highest encapsulation by this matrix, also had shown the most controlled release of drug molecule. The hydrogel with highest encapsulation had shown controlled release because the drug molecules were mostly encapsulated by absorption within the matrix. Further this hydrogel has better adhesion property and highest cell viability amongst different mole compositions of AA content.

Chapter 4 involves the synthesis and characterisation of a newer biomaterial from CMT polysaccharide by grafting with HEMA. The detailed synthetic protocol is similar for the synthesis of TKP-g-AA hydrogel except the initiator used in this case was benzoyl peroxide (Detailed discussion in Chapter 3). The purpose of switching over to carboxymethylated tamarind polysaccharide from TKP as base polymer was to tap the advantages CMT such as better water solubility and stability of biopolymer from microbial degradation, however without compromising the basic advantages associated with TKP. It also provides functionality diversification by introducing carboxylic group in addition to –OH functionality thereby adding to the functionality variability to the biopolymer. Needless to mention here that TKP has certain inferior material quality as compared to CMT with respect to solubility, thermal stability and resistance against microbial degradability [Sen et al. 2009; Goyal et al. 2007]. Polysaccharides are generally water soluble [Rana et al. 2011], and these properties of extreme water solubility and/or susceptibility to degrading enzymes make them less suitable for use as solid scaffolds/matrices. Therefore, proper hydrophilic-hydrophobic balance of a hydrogel is an important criterion suitable for their biological applications. In case of CMT, the –OH moieties are not able to form strong ionic interactions with counter ions [Rana et al. 2011; Hoare et al. 2008] that limits their effective biological
applications. This necessitates further custom-made improvement of CMT-based matrices for specific applications. In order to convert CMT suitable for biological applications, potentially different functional groups have been introduced \[Rana et al. 2011\]. Previously it was observed that different groups such as carboxyl groups, carboxymethyl groups, polyacrylamide groups, phosphate groups etc increases its hydrophobicity \[Rana et al. 2011\]. In this work the CMT was modified by grafting with HEMA.

This work demonstrates that grafted CMT-HEMA at a ratio of 1:10 had shown good material properties for adhering and further growth of bone precursor cells (RAW 267.4) suitable for bone tissue engineering. This work also demonstrated that different sensitive cells such as bone precursor cells, neuronal cells and Human Umbilical Vein Endothelial cells (HUVEC) grown successfully on this hydrogel surface. However, the growth pattern is largely different for different cell types ranging excellent growth to moderate growth for some cells. These also indicate that the surface and material properties are optimum for other cells and ideal if not excellent for preosteoclast (RAW 264.7) cells and also for the HUVEC cells. The bone cells have been reported to be extremely sensitive in terms of their surface requirements as cell adhesion-mediated signalling events play a major role in the survival and chemotaxis events \[Lee et al. 2006\]. Non-compatible surfaces can induce apoptosis in osteoblasts and cause major bone defects \[Grigoriou et al. 2005\]. Preosteoclast cells studied in this work proliferated easily on this surface for a long duration without any symptom of cytotoxicity and form dense clusters (till 100 hours as observed in this study) indicating that this surface offers positive adhesion and focal signalling events relevant for preosteoclast cell adhesion and further growth. These events were ascribed to the compatible properties of the cell adhesion molecules, specific receptors, ion channels and intrinsic affinity of these cells for complex carbohydrate molecules. In agreement with this, we demonstrate that the preosteoclast cells adhere well on this surface and their filopodial structures are well spread. The focal adhesion points are distinct. This observation is in accordance with several reports suggesting that integrin- and focal adhesion-mediated signalling events play key roles in bone cell adhesion, chemotaxis, growth, differentiation and maturation \[Carvalho et al. 2003; Cheng et al. 2001; Damsky 1999; Kim et al. 2007; Nakayamada et al. 2003; Salasznyk et al. 2007\]. The early events of cell adhesion and further growth also suggest that this surface is better than few other surfaces prepared before where cell adhesion is either poor and takes long time or the surface exerts cytotoxicity \[Lai et al. 2010\].
This chapter demonstrates from different characterization that the hydrogel with 1:10 mole ratio of CMT with HEMA has resulted in best hydrogel with highest grafting of HEMA on to the CMT backbone. The work also concludes that the hydrogel can be potential biomaterial for application tissue engineering and for controlled drug delivery devices. Successful attachment and growth of different cells such as Neuro2a, RAW 264.7, and HUVEC cells on this surface also suggest that this surface can also be used in Human and can be used to induce cell and tissue differentiation. However, further human bone cell specific studies are required in future to assess the clinical potential of this biomaterial.

The in vitro drug dissolution study had demonstrated that the hydrogel with highest grafting of HEMA on CMT backbone (CMT-g-HEMA with 1:10 mole composition) shown highest encapsulation efficiency as well as most controlled release pattern for paracetamol. In contrast to TKP-g-AA hydrogel matrices, wherein the matrix with moderate hydrophilicity had shown highest encapsulation efficiency and most controlled release efficacy, the CMT-g-HEMA hydrogel with least hydrophilicity amongst different compositions of CMT and HEMA had shown the highest encapsulation efficiency and controlled release efficacy. The material efficacy of CMT-g-HEMA (1:10 mole composition) in the context of adhesion and growth of different cells also had shown equally good biophysical properties. Thus it can be concluded that this material can have various potential biomedical application in controlled release devices and tissue engineering.

Chapter 5 involves designing of a ternary hydrogel by incorporating both the pendant groups that are used in synthesis of TKP-g-AA and CMT-g-HEMA with the aim optimising properties associated with these two pendant groups. In the preceding section (Chapters 3), the role of hydrophilic monomer in functionality modification of TKP has been investigated. In chapter-4, the incorporation of hydrophobic pendant and the consequent functionality transformation have been investigated. The impact of functionality modification of polysaccharides on cell adhesion and growth of different cells has been studied.

The Chapter 5 involves assessment of biophysical properties of ternary hydrogel with compositional variation. The hydrogels were analysed for cell compatibility by MTT assay. Different cell lines (RAW 267.4 and Saos-2) were grown on this hydrogel to assess its potential biomedical applications in tissue engineering and drug delivery devices. The incorporation of dual monomer has altered hydrophobic and hydrophilic balance in CMT polymer backbone which has influenced physico-chemical and biophysical properties. It was
observed that the cell viability has increased for hydrogels (S1, S3 & S9) with hydrophilicity. The AA incorporation substantially increased the cell viability (ca. 100%), however in case of relatively less hydrophilic hydrogels (S2 and S4) the cell viability (∼80%) is comparable to pure CMT. The decrease in cell viability can be ascribed to relatively hydrophobic character due to HEMA incorporation. The hydrogel was further studied for controlled drug release ability with hydrophilic paracetamol as a model drug. It is demonstrated here that the hydrogel (S9) with moderate hydrophilicity (swelling 1057%) amongst the ternary systems shows most promising controlled release potential for paracetamol (Fig.7.1a-b). However the hydrogel (S3) with highest swelling ability (2361%) shows faster release kinetics for paracetamol. It can be concluded that hydrophilic and hydrophobic character affects the release kinetics and thus hydrophobic-hydrophilic balance is quintessential characteristics that decides the controlled release behaviour in ternary hydrogels. All the ternary hydrogels had shown a sustained release of drugs upto 20 hours.

**Figure 7.1** a) Sustained release of paracetamol by ternary hydrogels for upto 20 hours, b) Swelling percentage of ternary hydrogels.

**Fig.7.2** shows a comparative release of drugs after 2 hours from different hydrogel based matrices. The hydrogel matrices with most controlled release pattern within their own group have been considered for comparative release analysis. The release efficacy of paracetamol from different hydrogel matrices shows a significant variation after initial release of 2 hours. The comparative plot shows TKP:AA at 1:5 mole ratio had produced the slowest release of paracetamol following highest encapsulation of the drug molecules by the hydrogel matrix (Fig.7.2). High encapsulation led to higher absorption of the drug molecule by the hydrogel molecule which eventually retained the drug molecule in TKP-g-AA hydrogel for longer time as compared to CMT-g-HEMA (1:10 mole ratio) and ternary S9.
hydrogel. However, regarding cell adhesion and growth of bone cells (such as osteoblast like Saos-2 and pre osteoclast RAW 267.4), all the hydrogels were found to be equally efficient and thus can be recommended as an excellent biomaterial surface for bone tissue engineering application.

![Figure 7.2 Comparative release of paracetamol from different ternary hydrogels.](image)

Chapter 6 involves the study for assessing the potential of CMT polysaccharide for the synthesis of silver nanoparticle by green synthetic methodology and exploration of the resultant silver polysaccharide nanocomposite for biomedical application particularly for anti-biofilm activity.

The TEM analysis had demonstrated the size of AgNPs to be in the range of 10-40nm and mostly spherical in shape. The CMT-capped AgNPs were free from agglomeration as demonstrated from unchanged SPR and Zeta potential value of -36mV. The AgNPs had demonstrated significant anti-bacterial activity against both gram positive and gram negative strains almost 100% killing at a dose of 175µM. Further, these CMT capped AgNPs, at a sub-lethal dose of 10 µM were found be very effective in inhibiting biofilm formation against both gram positive and gram negative strains. The cell viability assay also demonstrates that at this sub-lethal dose and even higher doses the mouse macrophage RAW 264.7 cells are significantly tolerant to the exposure of CMT-capped AgNPs. The CMT capped AgNPs were found to be very effective as these AgNPs do not affect the mammalian cells significantly at a concentration where it can achieve significant anti-microbial activities. It was found that the
chemical synthesised AgNPs didn’t show any anti-microbial activity at comparable doses whereas the green synthesised CMT capped AgNPs had shown effective anti-microbial activities (Fig.7.3).

Figure 7.3 Anti-microbial efficacy of CMT-capped AgNPs. a-b) CMT-capped AgNP concentration-dependent growth inhibition of Gram negative E. coli and Gram positive B. subtilis on LB agar plates, c) Chemically synthesized (NaBH₄-reduced) AgNPs are ineffective against the growth of E. coli and B. subtilis at the same concentrations.

Thus in a nutshell, it could be said that, in this thesis, attempts have been made to modify the tamarind based polysaccharide to different matrices by various functionality modifications by either introducing synthetic monomer by graft polymerization or embedding in situ synthesized nanosilver. Four different polysaccharide based matrices were developed and well characterized for different physico-chemical and biophysical properties. The matrices have shown promising biocompatibility with different sensitive cells. Further the matrices with optimal functionality modification have shown controlled drug release for hydrophilic drugs. Thus it can be ascertained that the materials have potential biomedical applications. Further investigation are required to fully establish the potential applications in different other biomedical field.
Future studies

The polysaccharide based matrices with different extent of grafting as well as type of grafted pendant group are found to be biocompatible with bone precursor RAW 264.7 cell and osteoblasts like Saos-2 cells apart from few other sensitive cells such as Neuro2a, HUVEC, F11 and MC3T3 E1 etc. The polysaccharide based matrices also have shown promising potentials for controlled release devices for hydrophilic drugs. However following future studies are required to tap their full potential as biomaterials.

- Studies required involving *in vivo* and clinical experiments to assess the potential of these polysaccharide based matrices for bone tissue engineering application.
- Studies required for up-scaling the material potential in pharmaceutical application for drugs.
- Controlled release efficacy can be exploited for the controlled release of hydrophobic drugs with necessary functionality tuning by optimisation of hydrophilic-hydrophobic balances in the matrices.