Chapter 3

3.1 Introduction

Natural polymers are most fascinating candidates and provide supports that exhibit many advantages like biodegradability, biocompatibility, nontoxic, high reactivity, low cost, easy availability and fabrication [116]. Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6-8 glucose units connected by α- 1, 4-glycosidic linkages and depending on the number of monomeric units, they are classified as α-CD, β-CD and γ-CD for 6, 7 and 8 units, respectively. They have toroidal shape with a lipophilic interior cavity and the hydrophilic exterior having hydroxyl groups on the outer surface [101]. The hydrophobic cavity of 5–8 Å diameter and 7.9 Å height in CD provide favourable host guest interaction leading to the inclusion complexes with the variety of molecules [117]. Due to low toxicity and immunogenicity, [100] CDs are extensively used in biomedical applications to form inclusion complexes with drugs through host guest inclusion and other related applications including; increased drug solubility, stability, [118] masking odors and taste [119], drug release [120] and permeability across biological barriers [121]. CD is having potential as a drug carrier vehicle for delivering drug at specific site and time from its tendency to form complex and acts as a functional carrier material [122]. Cyclodextrin as an oligomer is an alternative material in biomedical arena but its applications are limited due to its brittle nature and water sensitivity, it cannot meet or replace the physical and functional properties of other polymers [123]. Thus, grafting on CD with other polymers can be an effective tool for attaining certain properties like sustained drug release by maintaining the hydrophilic-hydrophobic balance. Zhang et al designed a β-CD functionalized hyperbranched

polyglycerol (HPG-β CD) of paclitaxel to achieve high drug loading capacity and aqueous solubility. These nanoparticles are biocompatible and a promising delivery system for hydrophobic drugs [124]. Drug release of dexamethasone through hydro gels of hyaluronic acid and β cyclodextrin is reported.[125]. Polyurethanes belong to an engrossing family of synthetic polymer with varying molecular designs *i.e.* condensation reaction between isocyanates, diols, diamines or chain extenders. Unique properties have made it one of the most promising polymers to be utilized for the variety of products like flexible and rigid foams, medical devices, sports goods, primer, adhesives, sealants, coatings, tough solids, elastomers etc. [126] Polyurethanes are multifaceted polymers owing to properties like biocompatibility and biodegradability leading to its applications in biomedical field as a biomaterial, elastomeric product and shape memory materials [127]. Till date there are numerous reports on grafting of polymers and lot of work is done on grafting of polyurethane for various applications [128]. Tuning of graft density as well as chain length of graft are the important features for graft copolymers for versatile applications [129]. Chemical combination of two biopolymers for sustained drug release is an useful tool for tunable properties and introduction of hydrophobic polymer like PLGA on dextrin (hydrophilic) backbone has been made showing relatively less control on the release of anticancerous drug (paclitaxel) while there is no report on side effect of the drug on other vital body organs [130].

Here in this chapter the designing of novel graft copolymer with polyurethane chains of varying graft density on the CD ring to make them thermally and mechanically stable materials to be used for biomedical applications especially in control drug delivery for cancer treatment. Grafting has been confirmed through spectroscopic methods and its

structural details are obtained from XRD and small angle neutron scattering studies to understand the varying architecture originated from grafting. Sustained release of drug has been demonstrated using graft copolymer with its full fledged cytotoxicity studies. Animal model experiment have been designed to show the efficacy of the sustained release by fabricating novel 'patch' using drug embedded in graft copolymer vs. drug embedded in CD or pure drug. Efficiency is shown not only in terms of suppression of tumor but also in the side effect caused by the exposure of drug. The results have been compared with conventional injection chemotherapy and better results with the newly developed materials have been demonstrated in animal model.

3.2 Results and discussion

3.2.1 Different architecture through grafting

A series of graft copolymers were synthesized with varying graft density of polyurethane prepolymer or degree of substitution to generate polymer with very different properties. The general reaction is shown in ***Scheme 2.1*** in experimental section where extent of grafting of PU on CD has been made to alter the hydrophilicity and architecture of CD. Grafting of PU on CD is done by reacting isocyanate terminated prepolymer with the hydroxyl groups attached with CD. ^{1}H NMR spectroscopy verifies the nature and extent of grafting and representative NMR spectra of graft copolymers are given in ***Figure 3.1a.*** The chemical shift of -OH proton is greatly affected and the appearance of new signal at δ=7.0 ppm (marked by '*a*' in the spectrum) indicate the >NH proton in urethane linkage (-NHCOO-), caused by the reaction between isocyanate group of PU and hydroxyl group of CD [131]. The intensity of this >N-H peak at ~7 ppm increases gradually with larger number of PU chains attached with a single CD molecule as evident from reaction condition leading to higher content of grafting. The strong signals corresponding to methylene protons of PTMG polyol (-CH_2O-) at δ=1.5 ppm and

HMDI diisocyanate (-CH_2N=) at δ=3.9 ppm also support the grafting of polyurethane onto CD ring ***Figure 3.1b*** [132]. An estimate of the degree of substitution (DS) / grafting has been made from the ratio of integral peak signal at δ=4.4 ppm (OH-1 from CD) and at δ=1.5 ppm (-CH_2- proton of PTMG). The DS is found to be the highest (77%) for the sample termed as 'CDgPU-H' while 40% value is measured for the other sample designated as 'CDgPU-L'. In this juncture, one may argue with the formation of graft against physical mixture of CD and PU. The absence of NMR peak at ~7 ppm in the blend of CD and PU confirms the formation of graft in the above mentioned samples (^{1}H NMR evidence is presented in ***Figure 3.1b***.Molecular weights of CDgPU-L and CDgPU-H are found to be 33k, and 114k, respectively, with relatively low polydispersity index of ~1.35. Further, a bimodal distribution is noticed in higher molecular weight graft copolymer (CDgPU-H) revealing the presence of two molecular entities arising from the slight crosslinked species in addition to regular high graft density copolymer. This is to mention that the molecular weight of the physical mixture of CD and PU exhibits almost similar to PU and thus, prove the grafting of PU chain in CD ring (***Figure 3.1c)***. The evidence of grafting / chemical tagging of PU on CD is also shown by the FTIR absorbance peak at 1720 cm^{-1} assigned for urethane >C=O band and 1540 cm^{-1} peak for the urethane >NH bending (***Figure 3.1d***) [133].

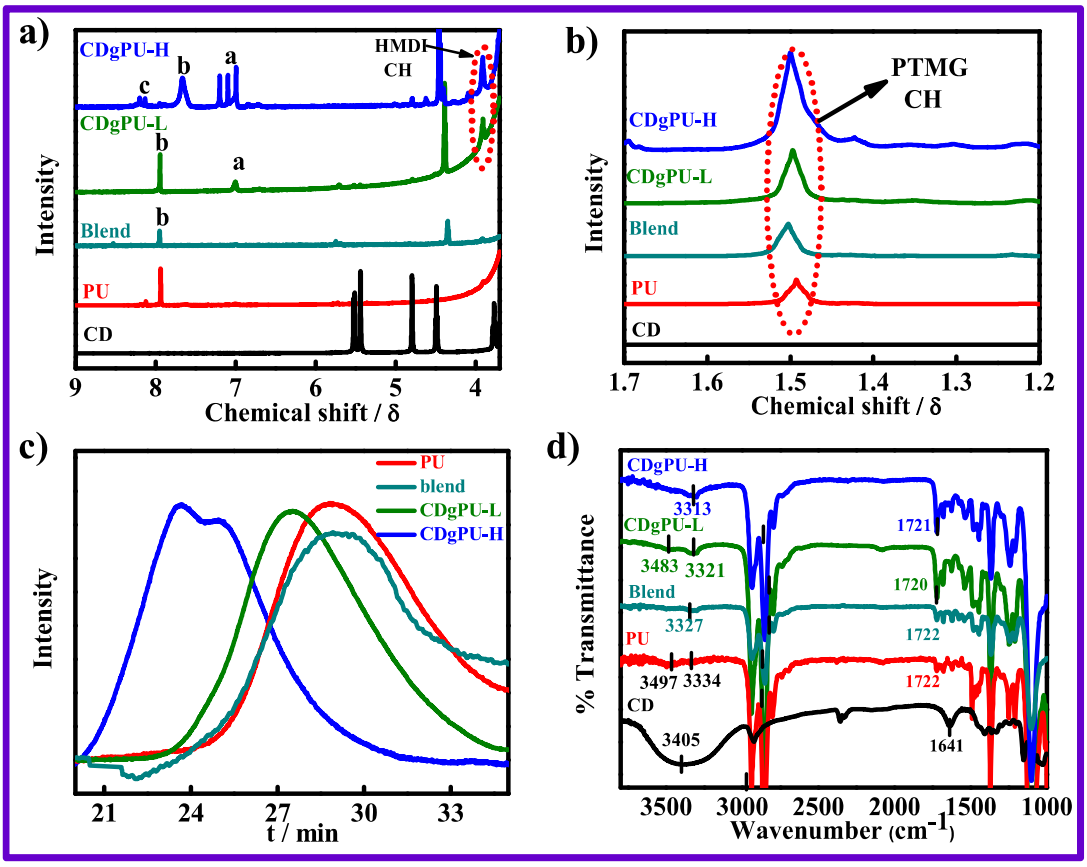

Figure 3.1: *a) ^{1}H NMR Spectra of CD, PU and their indicated grafts showing new peak position due to grafting marked by 'a' and change of hydrogen bonding as presented marked by 'b' b)NMR spectra of CD,PU and its respected graft showing appearance of new peak at 1.5 ppm attributed to proton of PTMG. ; c) Gel permeation chromatogram of graft copolymers showing lower elution time for high graft density copolymers d) FTIR spectra of pure CD, PU and their graft copolymers as indicated.*

Graft copolymers exhibit hydrogen bonded >C=O peak at 1680 cm^{-1}, also observed in pure PU, while its intensity decreases considerably with increasing degree of substitution indicating the meager intramolecular hydrogen bonding in higher substituted graft copolymers (CDgPU-H). A slight shifting in free >C=O peak is observed in graft copolymers due to interaction with surrounding polar group like –N-H. The broad peaks in the region of 3300−3400 cm^{-1} is due to –O-H stretching of pure CD which becomes narrower as well as shifted to lower wavenumber in graft copolymers. Further, the peak in this range splits into two peaks at 3497

and 3334 cm^{-1} for free and hydrogen bonded >N-H stretching, respectively, for pure PU which have shifted to 3313 cm^{-1} in CDgPU-H due to extensive intermolecular hydrogen bonding between PU chains in high graft density copolymer as opposed to doublet peaks at 3321 and 3483 cm^{-1} in CDgPU-L, a low graft density copolymer, where interaction of grafted PU chain with CD (intramolecular interaction) is predominant. FTIR spectra of pure CD with all the characteristic peaks, and blend system are presented in ***Figure 3.1d***.

UV absorption peak for CDgPU-L has shifted to lower wavelength of 277 nm as compared to pure PU absorption peak at 283 nm [134], assigned due to $n \rightarrow \pi^*$ Transition (***Figure 3.2a***). The blue shift in graft copolymer is attributed to the constraint under wrapping of polyurethane chain around CD ring. Now, it is pertinent that different graft density of PU on CD cause different architecture as shown in ***Figure 3.2b*** as measured from the calculation of graft density from NMR followed by the matching with molecular weight information. Interesting to note that molecular size increase with the grafting of PU while high density generate open structure as opposed to coiled structure in low graft density copolymer as explained earlier due to change of the nature of interaction (in case of CDgPU-L predominant interaction between PU chain and CD (intramolecular)) while intermolecular interaction takes place in CDgPU-H through hydrogen bonding between PU chains of neighboring molecules. However, wide varieties of graft copolymers with the change of interacting behaviour is helpful in designing biomaterial for delivering biologically active molecules by altering the hydrophilic-hydrophobic balance.

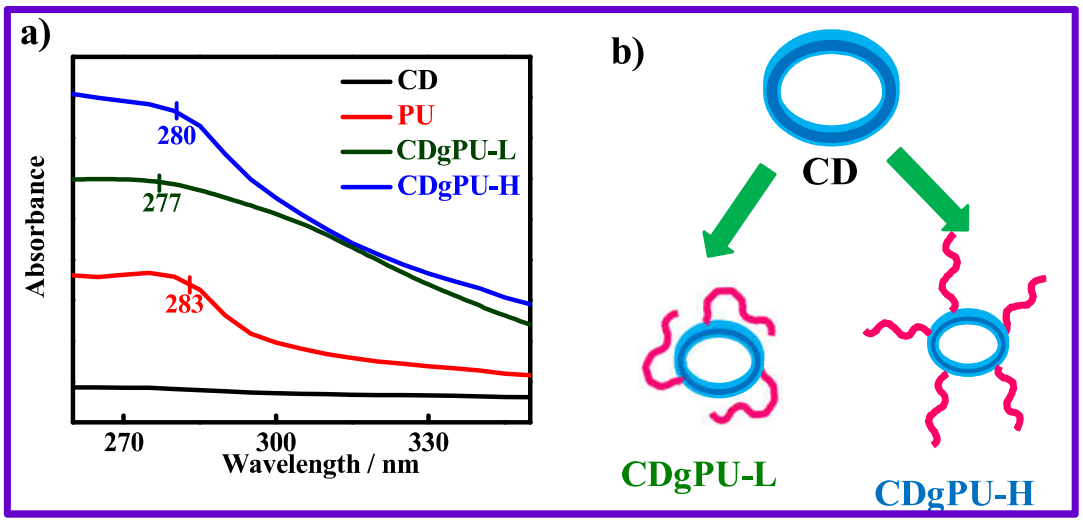

Figure 3.2: *a) UV- Vis Spectra of pure and graft copolymers. Vertical lines indicate the peak position for $n \rightarrow \pi^*$ transition of carbonyl peak; and b) Schematic presentation of the architecture of the graft copolymers with varying graft density. It also indicates the type of interaction present in different systems as appeared from coiled to open architecture arise from intramolecular to intermolecular interaction, respectively.*

3.2.2 Thermal and mechanical responses with structural alteration in graft copolymer

Pure α-cyclodextrin (CD) starts decomposing its glucose unit at ~300 ^{o}C apart from its slight weight loss at 100 ^{o}C due to evaporation of adsorbed moisture [135] while the thermal stability of graft copolymer increases by 50 ^{o}C after grafting with PU chain (***Figure 3.3a***). The degradation temperatures of CDgPU-L and CDgPU-H are found to be 348 and 343 ^{o}C, respectively, showing better thermal stability of grafted systems as compared to CD. Pure prepolymer (PU) shows a higher degradation temperature of 374 ^{o}C and wrapping of thermally stable PU over CD enhances the thermal stability of the graft copolymers. The degradation temperature was measured using thermogravimetric analyzer under nitrogen atmosphere and the temperature corresponding to 5% weight loss is considered as the degradation temperature.

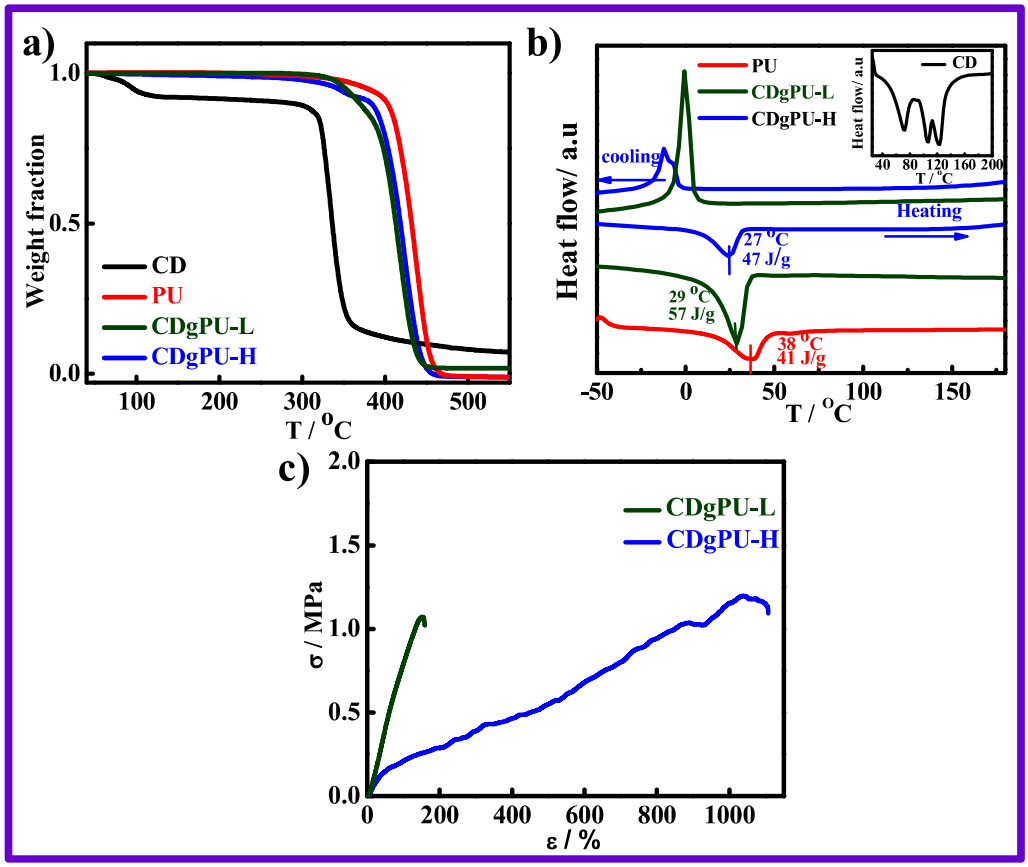

Figure 3.3: *a) Thermal stability (TGA thermogrammes) of pure CD, PU and their grafts as measured through thermogravimetric analyser; b) DSC thermogrammes of PU and its graft copolymers as indicated both for heating (bottom three) and cooling cycle (top two thermogrammes). The inset figure represents DSC pattern of pristine CD; c) Stress–strain curves of graft copolymers showing elongation at break.*

Further, pure PU shows single endothermic peak at 38 °C due to its melting of soft segment [136] while the graft copolymers exhibit single endothermic peak at 29 and 27 °C for CDgPU-L and CDgPU-H, respectively (***Figure 3.3b)***. Significant lowering of melting temperature in graft copolymer indicate strong interactive nature as discussed before while relatively lower heat of fusion (ΔH_m) in CDgPU-H (47 $J.g^{-1}$) vis-à-vis CDgPU-L (57 $J.g^{-1}$) further consolidates the strong intermolecular interaction in high density graft copolymer in CDgPU-H against comparatively weaker interaction with CD molecule (intramolecular) in

low graft density copolymer (CDgPU-L). This is to mention that lower melting temperature along with lesser heat of fusion is the indicator of strongly interactive system [137, 138]. Crystalline nature of CD is shown in the inset of ***Figure 3.3 b*** which is suppressed considerably in graft copolymer presumably due to lower abundance and wrapping up with PU moieties. Moreover, delayed crystallization in CDgPU-H over CDgPU-L during cooling also suggests relatively stronger interactive system in high graft density copolymer than that of low density graft copolymer (two above thermogrammes in (***Fig. 3.3b***). However, better thermal stability and stronger interaction is evident from the thermal measurements.

To visualize the effect of degree of substitution of PU on the mechanical properties of graft copolymers, uniaxial tensile measurements have been carried out to understand the stiffness and toughness of the graft copolymers. Interestingly, the elongation at break substantially increases (more than 1000%) with increasing the degree of substitution in CDgPU-H predominantly from its greater amorphous content while the stiffness of CDgPU-L is considerably high arises from its higher crystalline content (***Figure 3.3c***) [139]. As a result, toughness values, area under the stress-strain curve, are 85 and 725 $MJ.m^{-3}$ for CDgPU-L and CDgPU-H, respectively, indicating very tough graft copolymer with high graft density. Moreover, strain induced hardening phenomena is clearly visible in CDgPU-H which appears from its hyperbranched type of structure which promotes stress transfer mechanism along with subsequent crystallization at higher strain. [133]. Elastic modulus/stiffness (calculated from the slope of the initial linear regime) is relatively high in CDgPU-L (7.42 MPa) as compared to CDgPU-H (5 MPa) presumably because of their comparative crystallinity as discussed in previous section. Higher crystallinity in CDgPU-L

is further substantiated from the XRD patterns where predominant amorphous structure is shown in CDgPU-H as opposed to crystalline peaks observed in CDgPU-L (***Figure 3.4a***). Pure PU and CD exhibit highly crystalline nature and they cannot be moulded in the dog shaped form and thereby couldn't be possible to measure the mechanical strength using UTM. For better understanding the grafting of PU onto CD ring, small angle neutron scattering of the graft specimens are performed showing a shoulder at the wavevector, q ~ 0.25-0.30 nm^{-1} corresponding to the characteristic length $\Lambda_c(=2\pi/q_m)$ of 22, 25 and 24 nm for pure PU, CDgPU-L and CDgPU-H, respectively, indicating that lesser number of molecules are required to form self assembly in highly grafted systems (***Figure 3.4b***). The correlation length, ξ as calculated from Debye-Bueche fitting of initial wavevector are found to be 1, 0.5 and 0.3 nm for PU, CDgPU-L and CDgPU-H, respectively, clearly demonstrate the smaller blob size in graft copolymer than that of pure PU. Polyurethane is known to form aggregates through hydrogen bond between hard segments and the blob size usually increases in presence of nanofiller in PU while the grafting over CD ring form different blobs through hydrogen bonds from grafted PU chains, thereby the number of blobs increase but their individual size decrease in CDgPU-L to CDgPU-H gradually. The size of inhomogenities in pure CD is close to the value of 2 nm and the bigger size is explained from the extensive hydrogen bonding amongst CD rings resulting larger blob size. However, SANS data satisfactorily supports the schematic structures of graft copolymers formed and their subsequent agglomeration. In brief, mechanical and thermal properties can be altered by varying the graft density of PU and thereby make them suitable for drug delivery vehicle with proper thermal and mechanical stability based on their unique structure.

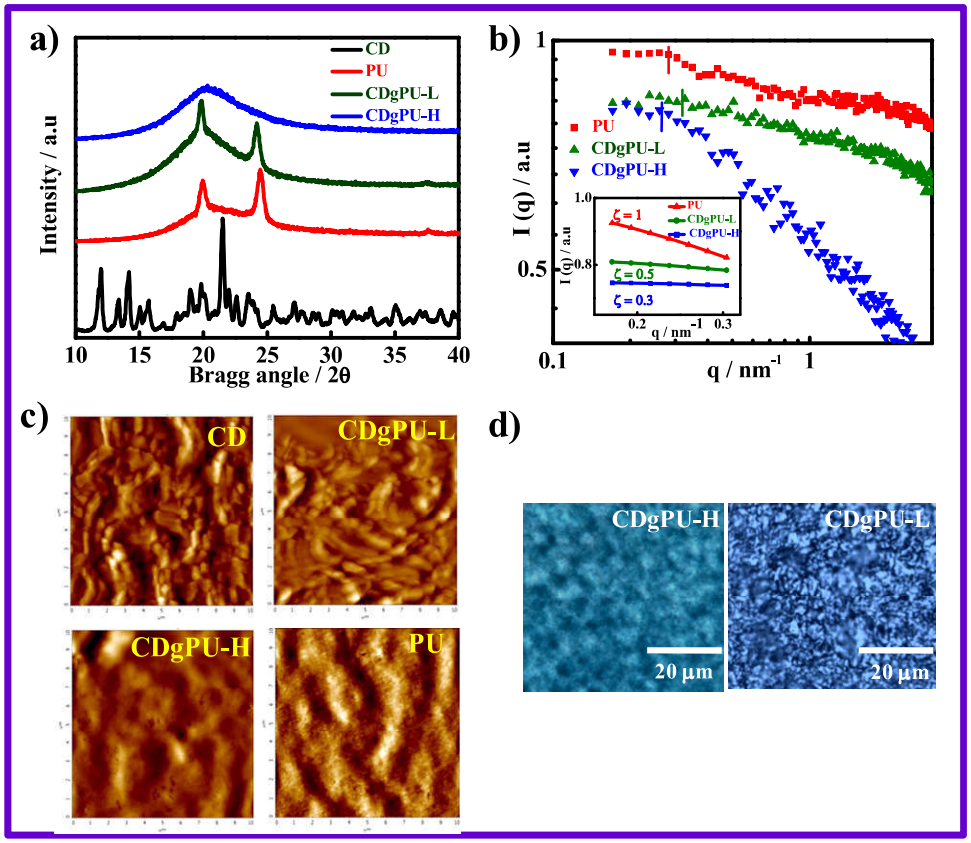

Figure 3.4: *a) XRD patterns of pure CD, PU and their graft copolymers; b) Small angle neutron scattering profile of PU and its graft copolymers. Inset figures represent the Debye-Bueche fitting of the initial data points showing the correlation length; c) AFM images of CD, PU and their grafts in semi contact mode (10×10 μm^2); and d) Optical images of the graft copolymers showing greater agglomerates.*

AFM imaging is well suited to visualize the surface topography of polymeric films along with the roughness and average size of the inhomogeinities. Granular particle of pure CD is observed with the average dimension of 400 nm (***Figure 3.4c***). Strip like morphology is noticed in CDgPU-L whose intensity has enhanced significantly in CDgPU-H presumably due to hydrogen bonded agglomeration in the hard segmented zone as discussed earlier. Stronger strip morphology is evident in pure PU arising from clustering of hydrogen bonded hard segment as reported earlier. There are self-assembly of hard domains to form larger

micro domains in CDgPU-H due to intermolecular hydrogen bonding. The alternate bright and dark strip patterns of domains arise from hydrogen bonded interactions between >C=O and >N-H groups which are responsible for the gradual change of morphology in graft copolymers. These images show a distinct phase separation between the dark phase (the softer matrix) regions surrounding the light-coloured *i.e.* hard segment especially in CDgPU-H [140]. Greater inhomogeinities of cluster is further noticed in optical images (***Figure 3.4d***) and larger dimension of cluster is observed in CDgPU-H (6 μm) as compared to CDgPU-L (2 μm). In this juncture, **s**elf assembly in graft copolymer is evident from the nanometer dimension of blob size (obtained from SANS fitting) agglomerate to form ~400 nm strip size captured through AFM image to 2-6 μm measured from optical images clearly indicate the greater self-assembly starting from very small size (nanometer dimension) to micron size predominantly through hydrogen bonding. This self-assembly may help in binding biologically active molecules like drug for their controlled release.

3.3 Sustained release of drug using varying architecture

Controlled release of drug is the prime requirement to regulate the concentration of drug in blood stream to obtain the highest efficacy of drug without side effect. 5 wt.% of dexamethasone drug is embedded in varying architecture of graft copolymers through solution route and its release profile in phosphate buffer solution (pH ~7.4) at 37 °C has been studied. The complete release of drug occurs in CD-d (drug embedded in CD) within an hour (burst release) while dramatic sustained release is observed in graft copolymer (***Figure 3.5a***).

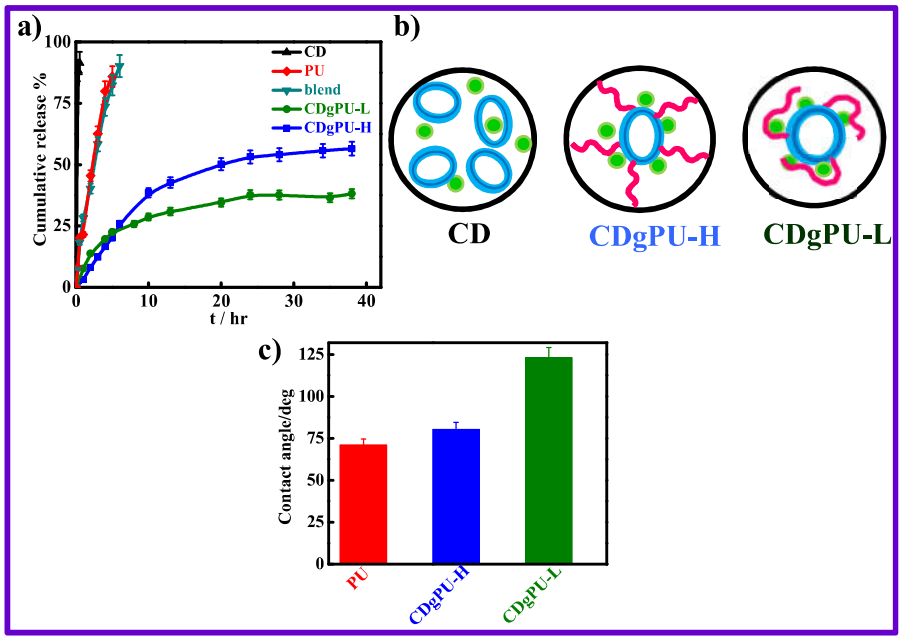

Figure 3.5:*a) Cumulative release of drug from the indicated specimens showing sustained drug release profile from graft copolymers; b) Schematic model showing the architecture of copolymer and drug which cause sustained release from low graft density copolymer against high graft density copolymer; c) Bar diagram of contact angle of various graft copolymers showing hydrophobic nature of low graft density copolymers as compared to high graft density copolymer.*

Low density graft copolymer (CDgPU-L) exhibits highest sustained release especially compared to high density graft copolymer (CDgPU-H). As discussed earlier, low density graft copolymer forms intramolculear hydrogen bonding with the attached CD molecules against predominant intermolecular hydrogen bonding in high graft density copolymer which causes relatively open structure in CDgPU-H as opposed to squeezed structure in CDgPU-L, which hold the drug molecules in a better way resulting sustained release. Based on this architecture, sustained release is understood and presented in a cartoon in (***Figure 3.5b)*** which explains reasonably well the relative drug release kinetics from varying architecture of graft copolymers. However, the burst release phenomena of pure CD is overcome through grafting it with suitable graft density of PU chain over CD

causing the release of 95, 56 and 40% of drug from pure CD, CDgPU-H and CDgPU-L, respectively, during 42 hours. Drug release from polymer matrix depends on various factors such as penetration of solvent into the matrix, dissolution of the drug and finally, the diffusion of drug from the matrix polymer. However, slower diffusion of drug is the rate determining step which is highly controlled by the wrapping of CD ring with PU chain and also by converting it to hydrophobic nature from the original highly hydrophilic nature of pure CD. Contact angle indicate the nature of hydrophilicity of a specimen and higher value of CDgPU-L (122^{o}) suggests hydrophobic character against considerably lower value of CDgPU-H (80^{o}) further consolidate the cartoon structure and resulting hydrophilic-hydrophobic balance, by controlling the graft density using PU graft, which eventually regulate the drug release profile from the polymer matrix (***Figure 3.5c***). The incorporation of a hydrophobic component on the polymer backbone disrupts the packing of polymer chain in the solution leading to pore formation and thus faster release of the drug occurs. The higher swelling of low graft density copolymer (CDgPU-L) as compared to high graft density (CDgPU-H) copolymer also suggest the greater hydrophilic character when the CD is grafted relatively less. Amongst these processes, any step can be the rate determining step of the whole drug release kinetics. Delayed diffusion of drug occurs in PU grafted CD as compared to pure CD. This is to mention that mixture of CD and PU (blend) shows similar burst release of drug like pure PU suggesting the importance of graft over just blend/mixture ***Figure 3.5a***.

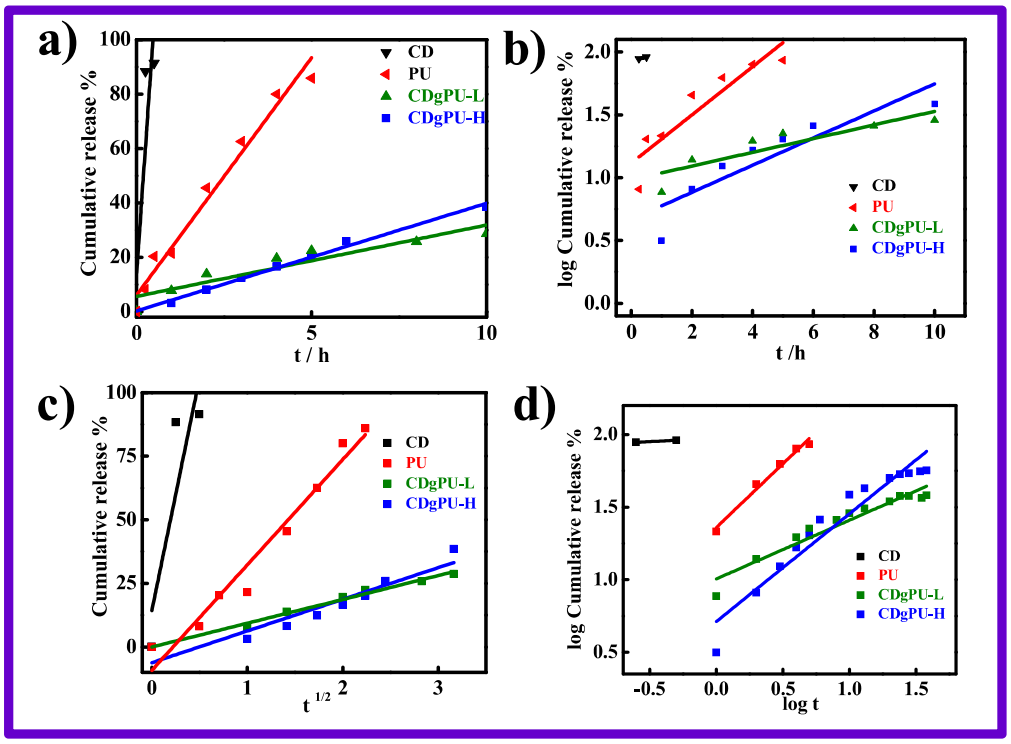

Figure 3.6: *Mathematical models for drug release kinetics. (a) Zero order model, (b) First order model, (c) Higuchi model, (d) Korsmeyer- Peppas model.*

Table 3.1: *Release constant k, correlation coefficient (r), release exponent (n) calculated from various models for drug loaded CD and its respective copolymers*

Sample	First order		Zero order		Higuchi		Korsmeyer Peppas	
	k	r^2	k	r^2	k	r^2	n	r^2
PU	0.1±0.03	0.81	17.14±1.1	0.97	41.45±0.32	0.95	0.87	0.97
CDgPU-H	0.1±0.02	0.71	4±0.12	0.99	12.4 ±1.6	0.89	0.74	0.92
CDgPU-L	0.05±0.01	0.73	2.6±0.45	0.85	9.3±0.45	0.98	0.40	0.92

To understand the release mechanism of drug, different models have been used to fit the release profile from various vehicles and the release kinetics is best fitted with Korsmeyer-Peppas model with higher linear correlation coefficient values (r^2 ~ 0.98) having the

exponent '*n*' values of 0.87, 0.74, and 0.40 for pure PU, CDgPU-H, and CDgPU-L respectively, indicating non-Fickian ($n \geq 0.45$) diffusion kinetics of drug molecules through all the systems except CDgPU-L **[141].** Other models of kinetics such as zero order, first order and Higuchi models are presented in ***Figure 3.6*** and ***table 3.1***. The interaction between drug and polymer matrix is one of the main reasons behind sustained release of drug and has been shown through spectroscopic and thermal measurements. Drug (dexamethasone) exhibits the characteristic absorption peaks at 273 and 340 nm while the peaks have been shifted to lower wavelength (blue shift) in CD-d (drug loaded CD) to 265 nm (***Figure 3.7a***). Further blue shift has been observed in CDgPU-L-d (drug loaded CDgPU-L) to 260 nm clearly indicate stronger interaction between drug and graft copolymer as compared to CD and drug interaction. Blue shifting of drug absorption peak appears from its confinement within the CD core or grafted CD core [142].This is to mention that relatively lesser shifting is observed in CDgPU-H-d (263 nm) suggest lesser interaction than that of low graft density copolymer (CDgPU-L-d). (***Figure 3.7b)*** compares the DSC thermogrammes of representative pure copolymer and drug loaded copolymers (CDgPU-L-d). The melting temperature of the drug loaded graft copolymers reduces to 18 ^{o}C from pure copolymer melting of 28 ^{o}C. The decrease in melting temperature arises from strong interaction between the components and the depression of ~10^{o} is considered as strongly interactive system.

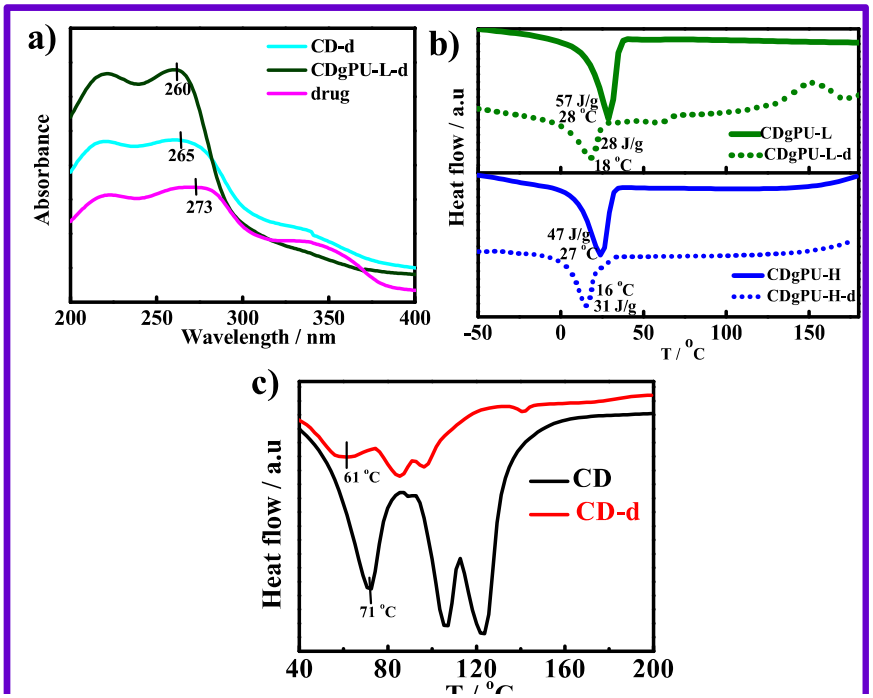

Figure 3.7: *a) UV-Vis spectra of indicated samples showing polymer-drug interaction. Vertical lines indicate the respective peak positions; b) DSC thermogrammes of representative CDgPU-L and its drug loaded specimen showing shifting of melting peak and heat of fusion in presence of drug; c) DSC thermogrammes of pure and drug loaded CD.*

Further, the heat of fusion (ΔH) for the said copolymer reduces significantly (28 $J.g^{-1}$) in presence of drug as compared to pure copolymer of 57 $J.g^{-1}$[143]. Similar reduction in melting temperature and heat of fusion is observed for other drug loaded copolymers. Slightly lesser reduction of melting temperature and heat of fusion are reported for high graft density copolymer suggesting relatively less interaction than that of low density graft copolymer with the drug. However, both the spectroscopic and thermal measurements indicate stronger interaction between drug and copolymer as opposed to drug and CD which is responsible for its slow release in grafted system as compared to CD-d (drug embedded CD) (***Figure 3.7c)***.

3.4 Biocompatibility and in-vitro efficiency under controlled release

It is important that the materials used for control drug delivery should be biocompatible and can have the efficacy to kill the infection / disorder by releasing the embedded drug in a regulated manner depending on the physiological requirement. The biocompatibility of the newly developed graft copolymers has been tested using cellular studies. To understand the biocompatibility, the viability of these cells over the surface of polymeric films has been studied through MTT assay with varying time intervals as shown in ***Figure 3.8a***.

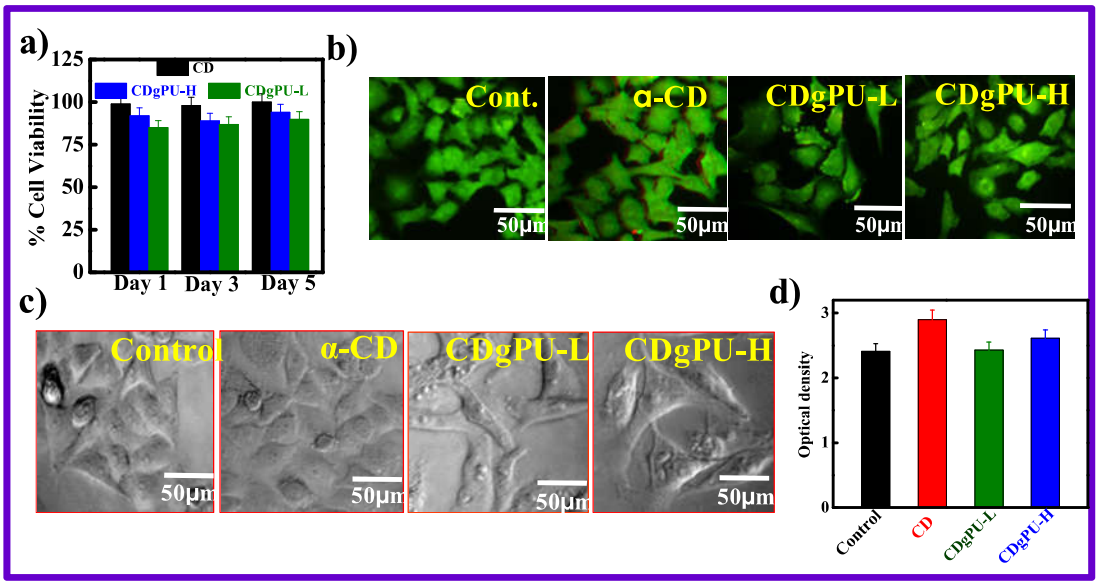

Figure 3.8: *Biological responses of graft copolymers through cellular studies. a) Cell viability of indicated samples at time interval of 1, 3 and 5 days through MTT assay measurement; b) Fluorescence microscopic images of cell cultured on indicated specimens and images are taken after one day of cell proliferation (mag 40×); c) Morphology of cells grown on different sample surface as indicated (cell adhesion); d) Quantification of cell adhesion through optical density measurement of adhered cells over various sample surface.*

MTT is reduced to formazan in the live cells by the enzyme mitochondrial reductase measured after 24, 48 and 72 h of incubation of HeLa cells on CD, PU and their graft

copolymers surfaces. CD and PU are known to be biocompatible [138, 144, 145] while no considerable decrease of cell viabilities is observed for graft copolymers. Time dependent MTT assay reveals the nontoxic nature of the graft copolymers and slight better compatibility of CDgPU-H over CDgPU-L is explained from their respective hydrophilicity which has also been confirmed from the cell morphology using fluorescence imaging of HeLa cell after staining with acridine orange and ethidium bromide (***Figure 3.8b***). Further, cell adhesion is understood to be the first step to consider any material as biomaterial because cell has to dock/adhered before growing over a sample surface. Cell adhesion and their morphology on top of the various graft copolymers suggest healthier cell morphology (elliptical) over graft copolymers than that of CD (***Figure 3.8c***). It is clearly seen that the number of Hela cells adhered to the surface of CD is more than that of CDgPU-H / CDgPU-L but the cell proliferation and cell health is much better as opposed to pure CD, where poor spreading of cells is observed. The number of adhered cells over sample surface is measured quantitatively after scraping out the cells from the respective surfaces and optical density indicate the relative presence of adhered cells (***Figure 3.8d***). Higher values of optical density for graft copolymers as compared to control indicate better adhesion of cells over graft copolymers [131, 146, 147]. Since polyurethanes are biocompatible in nature and are being used as an implant material in biomedical devices, these chemically grafted copolymers are expected to be better option with its enhanced physical properties together with greater biological activity in terms of cell viability and adhesion. Hence, the cell study elucidates that developed graft copolymers induce no stress on cultured cells and they are safe to be used for biomedical applications including drug delivery. Sustained anti-cancerous in-vitro drug (dexamethasone) delivery is reported using

graft copolymers in the previous section. The drug is embedded in various graft copolymers and the matrix copolymer is biocompatible in nature. Now, it is pertinent to understand the effect of control release of drug using the developed material on cell line, or, in other words, how the control release help killing the cancerous cell (HeLa cell line). This is to mention that concentration of drug also plays an important role on cell mortality/viability of Hela cells as monitored after incubation with varying concentration of free drug and similar drug loaded in copolymers in the range of 10 - 500 μg/ml and are reported in ***Figure 3.9a &b***. Cell health is monitored as a function of time (1, 3 and 5 days after incubation) keeping the optimum drug content same in all the cases (500 μg/ml) and pure drug concentration of 500 μg/ml is used for negative control. Initial cell mortality is high (~56%) for pure drug and drug embedded CD while initial viability is high for drug embedded graft copolymer (***Figure 3.9c***).

Interestingly, cell viability gradually decrease with time and reaches as low as 20 % (cell mortality of 80%) after 5 days for graft copolymers while systematic increase in cell viabilities are noticed for pure drug and drug embedded CD treated cells. Pure drug and CD-d (drug embedded CD) instantly expose to the media causing immediate cell death and the drug is consumed quickly in the media (within a day). So, the cell viability starts increasing in subsequent measurement both for pure drug and CD-d. On the other hand, initial cell mortality is relatively low (38 %) for drug embedded graft copolymers, because of less amount of drug is released within a day (sustained release), while significant mortality rate increases (81%) subsequently up to 5 days, mainly due to sustained delivery of drug for longer period of time as observed in-vitro drug release study (***Figure 3.5a***). However, at the end of 5^{th} day, the cell viabilities are 73 and 20% for pure drug and drug

embedded in graft copolymer, respectively, clearly indicating the efficacy of the sustained release of drug using graft copolymer against pure drug or conventional drug embedded in CD system.

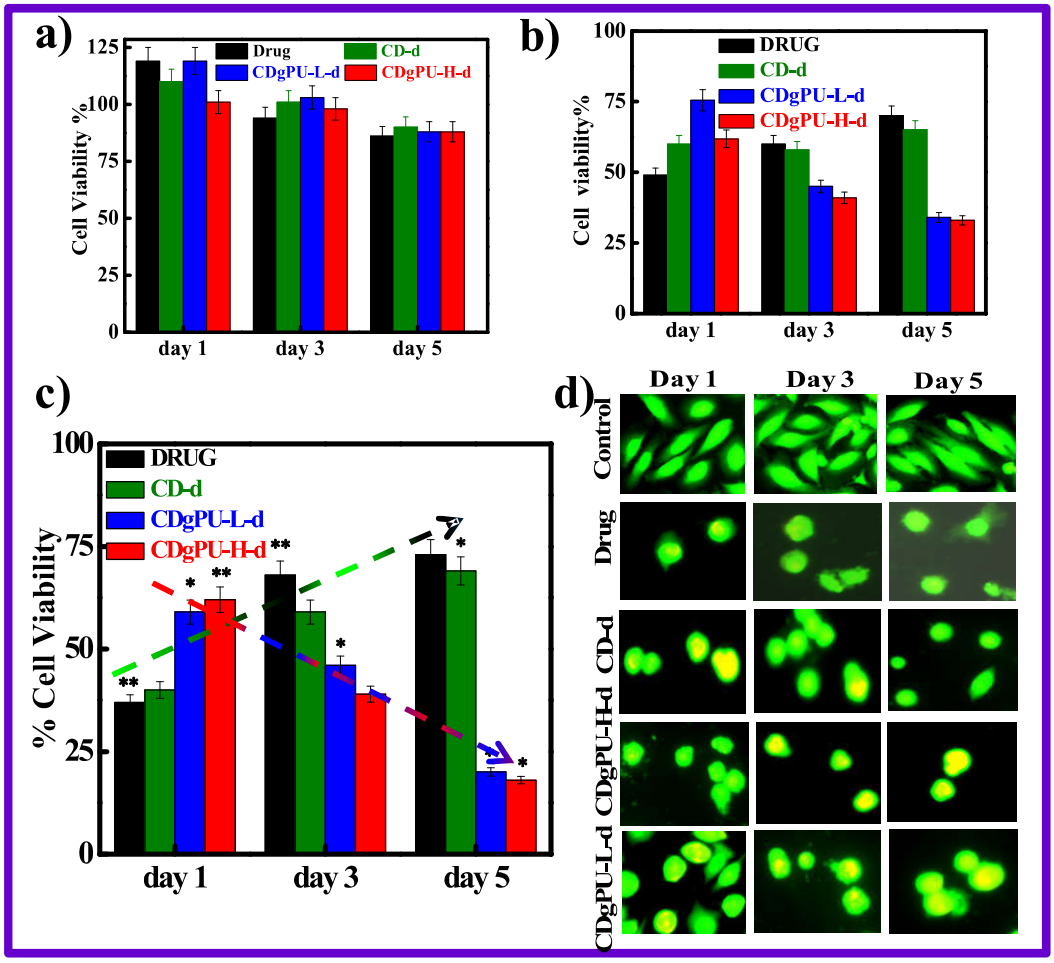

Figure 3.9: *In vitro cytotoxicity at different drug concentration: a) 10 µg/ml; b)100 µg/ml c)500µg/ml; d) Fluorescence images after AO/EB staining of cells treated with pure drug and drug loaded graft copolymers as mentioned(500µg/ml).*

The relative cell viability of drug loaded graft copolymers and the pure drug is also confirmed via fluorescence imaging of Hela cells during the cell culture process. The

morphological changes are analyzed using the double-staining technique (AO/EB staining) and are examined using a fluorescent microscope. In general, dead cells are permeable to both acridine orange and ethidium bromide and appear in red in fluorescence image whereas live cells are permeable to acridine orange only and thus show green colour in fluorescence image [148]. Healthy cells are observed in control system up to 5 days of treatment whereas less in number and squeezed apoptotic cells are noticed in all drug treated systems (***Figure 3.9d***). Apoptotic cells are visible in day 1 of the cells treated with pure drug and CD-d while healthy cells are observed in day 5 presumably because of fresh cell proliferation as also evident from cell viability data (***Figure3.9c***). On the other hand, drug embedded graft copolymers exhibit apoptotic cell in day 5 which clearly supports the very low cell viability at longer time, though the cell appears to be normal but stressed in day 1. However, cellular studies illustrate the higher killing efficiency of cancer cells by drug loaded graft copolymers as compared to pure drug and more than 80% cell mortality is observed in just 5 days. This is just to mention that the amount of pure drug and the total content of drug in each copolymer are kept constant for these comparative studies.

3.5 Efficacy of controlled release for cancer treatment in animal model

Encouraged by the in-vitro sustained release and its efficiency on the mortality of cells, in-vivo experiment has been performed to verify the therapeutic performances in animal model using the developed graft copolymers, here low graft density drug loaded CDgPU-L is used for animal model for both the treatment either patch or intravenous injection since it exhibited sustained release in better way. Definite size of the tumor has been generated by injecting 1×10^6 number of B16 F10 cells subcutaneously on the right flank of mice. The

mice are randomly distributed into four groups, each having 5 mice, when a palpable tumor of volume 30±5 mm^3 is developed. We design three different patches using drug embedded graft polymer (CDgPU-L-d), pure drug and drug embedded in CD (CD-d) separately to treat the tumors to understand their relative efficacy. All the animals were treated with patch made up of various systems including blank (treated with saline). The generation of tumor in mice and their relative size as a function of treatment time has been shown in ***Figure 3.10a***. Tumor volume significantly increase to 120% in blank system while meagre ~11% increase takes place in mice treated with the patch (drug embedded in low density graft copolymer) followed by its decrement in size after 18 days of treatment (***Figure 3.10b***).

On the other hand, patches with pure drug or CD-d exhibit relatively higher tumor volume than that of patch made of drug embedded in graft copolymer. Drug excreted from all the patches help recovering the tumor while sustained and prolonged release from the graft copolymer facilitates better healing of tumor and ultimately indicates the efficacy of graft polymer vis-à-vis pure drug or CD-d. Body mass index also supports the relative healing of tumor treated with varying patches and considerable weight loss is observed in control against mass gain for the mice treated with the patch made of graft copolymer material (***Figure 3.10c***). This is to mention that the patches were replaced on every third day and apparently there are no big differences in treatment between pure drug/CD-d and drug embedded graft copolymer. Higher body mass and lower tumor volume clearly differentiates the efficacy of graft copolymer over pure drug or drug embedded in CD. However, anti-tumor activity of the patch made of graft copolymer and drug has been verified.

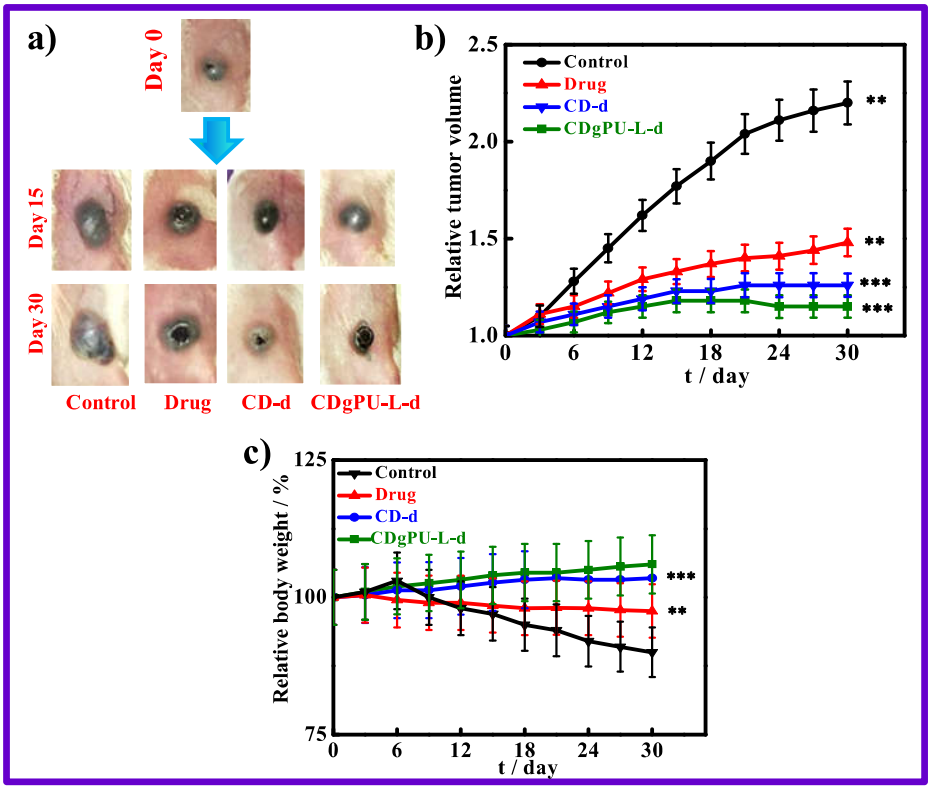

Figure 3.10: *a) Images of mice after tumor generation using B16 F10 cell line and tumor size after treatment with drug loaded indicated systems for varying time interval; b) Relative changes in tumor volume with time treated with indicated patches; c) Relative body weight percentage change as a function of treatment time.*

To reveal the real efficacy of the graft copolymer, the analyses of the internal organs and biochemical parameters of blood have been conducted after the completion of the treatment (after 30 days of treatment). Histopathology of various organs is performed to measure the effect of drug on other vital body organs (*e.g.* liver, kidney, spleen and tumor tissues). The organ tissues are excised and embedded in paraffin followed by staining of the thinner sections using H & E for histological analysis to evaluate any potential toxicity. Micrographs of all the organs are presented in (***Figure 3.11a)***.

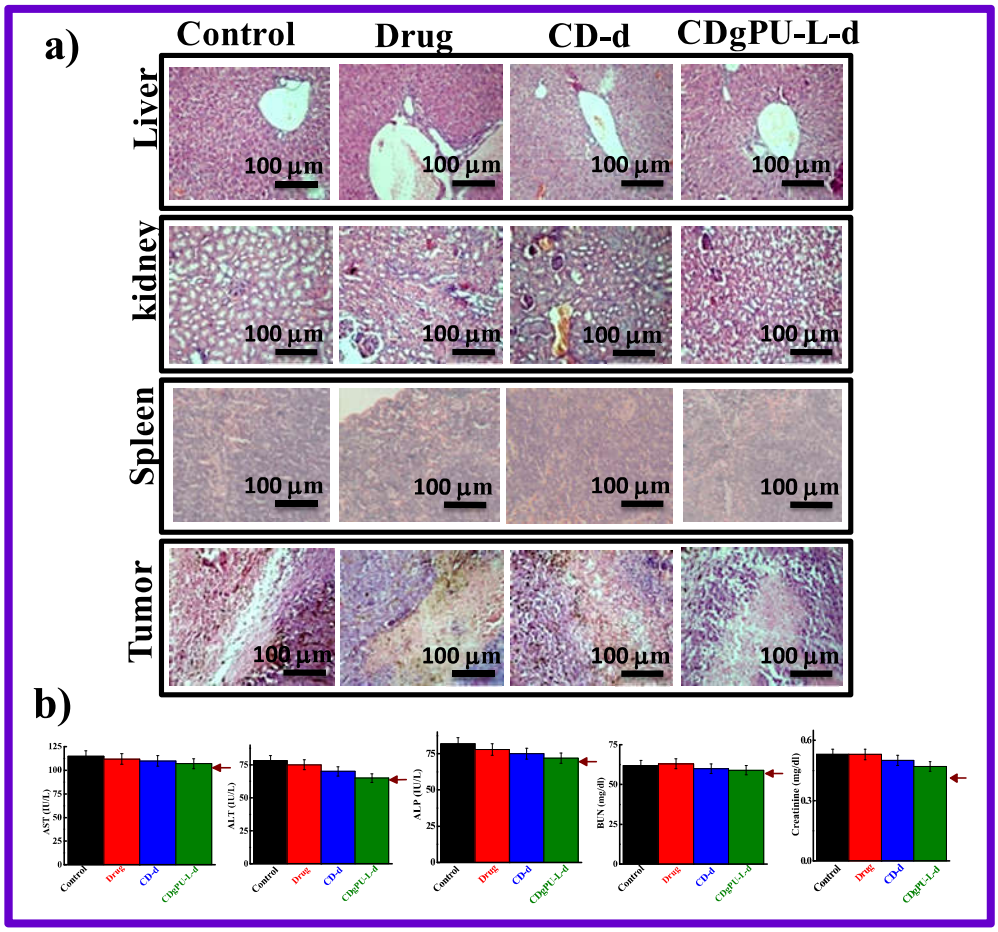

Figure 3.11: *a) Histopathological analysis of organs like liver, kidney, spleen and tumor dissected from mice after 30 days of treatment and b) Biochemical parameters, Hepatic function test including ALT, ALP, AST and renal function namely BUN and Creatinine of the mice treated with indicated patches. The arrows indicate the corresponding values of healthy mice [150]. All the results presented are mean ± standard deviation (SD) values obtained from three independent experiments, where *P <0.05, **P< 0.01, and ***P <0.001.*

Images of mice administered with pure drug exhibit marked damage in liver showing perivenular inflammation in portal tract with mild lobulitis while the control, where no treatment was given, display normal morphology, lobular arrangement, and mild lobulitis. Mice treated with CD-d shows loss of lobular arrangement and anisonucleosis of hepatocytes. However, no such toxicity is observed in the liver of the mice treated with drug embedded graft copolymer patch and normal morphology is maintained. Further,

degeneration of epithelial cells showing a cloudy appearance is noticed in mice treated with pure drug. Kidney of the mice treated with pure drug shows a slight focal tubular injury in epithelial cells while glomerulus, tubules and interstitium are unremarkable in control. The mice administered with drug loaded graft copolymer show no obvious kidney toxicity and displayed normal kidney morphology. No considerable morphological damages and lesions are observed in stained sections of spleen for all the systems.

Tumor tissues excised from the mice administered with pure drug show sufficiently higher necrotic spots necrosis. Histological analysis of the tumor in the mice treated with drug embedded in graft copolymer demonstrates necrosis with extensive nuclear shrinkages and fragmentation and these areas are sufficiently large among all the tested groups while the pure drug and CD-d display relatively lower necrotic zones. Microscopic examinations of tumor in control group show highly vascular and well-maintained integrity with some necrotic regions due to rapid tumor growth. The release of melanin from melanocytes due to breakage of melanosomes and cell death imparts reddish brown colour in tumor sections [149]. It is now evident that pure drug is exposed directly to blood stream in more quantity, assumed from burst release, in short time and affect the organs like liver and kidney by exceeding the therapeutic window while sustained release from CDgPU-L graft copolymer significantly reduces the excess concentration of drug at any time and thereby minimizes the side effect from the same amount of drug. However, histopathological results indicate that drug loaded graft copolymer CDgPU-L system protects the other vital body organs like liver from injury caused by immediate exposure of pure drug. Further, drug-loaded graft copolymer leads to a significant reduction of tumor growth without affecting the health of

the other body parts and thereby the newly developed materials overcome the side effect of conventional chemotherapy treatment.

Biochemical analyses of blood serum play an important role in the evaluation of organ functioning. Blood biochemical analyses are performed to unveil any toxic effects of drug, if any, after the treatment of melanoma bearing mice with anti-cancerous drug. Hepatic and renal functioning analyses including parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine are the prime indicators of the animal health and the data is presented in (***Figure 3.11b)***. Mice are treated with pure drug, CD-d and drug loaded CDgPU-L copolymer patches at the same dose of drug in each case and the blood sample is collected on 30^{th} day after the completion of the treatment.

Control group of mice exhibit highest level of all the biochemical parameter while drug loaded CDgPU-L copolymer shows lowest level under similar treatment. This is to mention that pure drug and CD-d treated mice exhibit higher values of all the parameter than that of CDgPU-L copolymer. The physiological values of AST, ALT and ALP in blood serum of healthy mice are 100, 60 and 70 IU/L, respectively, shown by the arrows in individual bar diagram [151]. The estimation of liver function is done from ALP, AST and ALT levels and ALT/AST ratio provides information about hepatic damage [152, 153]. The ratio of ALT/AST of mice treated with pure drug is ~0.66, considered to be high, while 0.60 value is measured for the mice treated with CDgPU-L patch (drug loaded graft copolymer) which is exactly similar to the value of 0.60 for healthy mice (indicated by arrows in (***Figure 3.11b)***. Higher activity of these two enzymes is in good agreement with damage to heart, skeletal muscles as well as parenchyma of liver (dysfunctioning of liver) which cause

disturbances in their syntheses in pure drug treated group or control [154]. However, elevated levels of all hepatic parameters in control group (ALT/AST ~ 0.67) along with pure drug treated mice group clearly demonstrate the hepatic damage due to uncontrolled growth of melanoma cells or crossing the therapeutic limit arising from burst release for pure drug or CD-d, thereby, affecting the organs significantly whereas the patch having drug loaded CDgPU-L copolymers does not affect the other body organs and restrict the growth of tumor in a controlled way. BUN and creatinine level in control mice group and the group treated with pure drug are slightly raised from the normal levels (indicated by the respective arrows in ***Figure 3.11b*** while almost normal values are obtained for the mice group treated with the patch comprising drug loaded in graft copolymer. Slightly high BUN and creatinine levels in control and pure drug treated group are attributed to renal dysfunction against no such symptoms observed in mice using the newly developed patch of dug loaded CDgPU-L.

The results demonstrate that the drug loaded graft copolymer, having required architecture, drug loaded CDgPU-L (patch) can efficiently deliver dexamethasone to melanoma tumor site inhibit cell proliferation and at the same time increase the apoptotic cells, resulting persistent suppression of tumor growth without any side effect. In this juncture, one may argue that conventional intravenous injection or in site injection may help with similar kind material which can deliver sustained release instead of patch as shown. In order to compare, we have performed the similar in-vivo experiment by injecting the drug, CD-d and CDgPU-L-d in solution form below the tumor site using separate mice group. The results are summarized in (***Figure 3.12).*** Tumor volume is slightly bigger in injected system than that of drug loaded CDgPU-L patch ***Figure 3.12a & b***) while almost similar

gain in weight is observed for both the cases (***Figure 3.12c)***. However, all the biochemical parameters including ALT, ALP, AST, BUN and creatinine have higher values in injected group of mice than the mice group treated with patch (***Figure 3.12d)***.

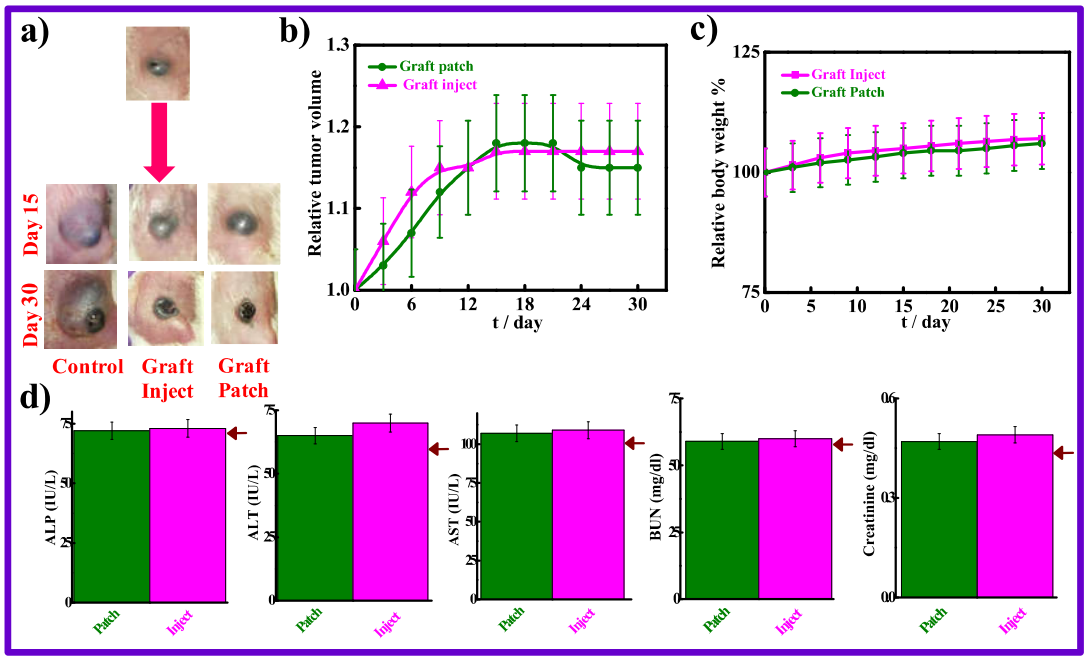

Figure 3.12: *Comparison of efficacy of graft patch vs. conventional graft inject system. a) Images of mice after 15 and 30 days of treatment with patch and injected systems; b) Relative changes in tumor volume with time; c) Relative body weight % of mice treated with graft patch and injection using similar composition; d) Biochemical parameter analysis including AST, ALT, ALP, BUN and CREATININE. The arrows indicate the corresponding values in healthy mice.*

This is to mention that amount of drug was kept exactly same for both the drug administered process. Hence, from the tumor volume and biochemical parameters, it is inferred that the efficiency of patch is marginally better and importantly there are less toxicity induced in the prime organs by using patch as the drug delivery vehicle instead of injecting the drug directly into blood stream. In the injection process, the material including

drug is carried away through circulatory system thereby the effective concentration in the tumor site is less as expected while the drug is being diffused in to the tumor tissue specifically directly from the patch, sustained release further insist the effective concentration throughout. On the other hand, as the drug is carried away through circulatory system, the anti-cancerous drug affect the other body organs resulting increased biochemical parameters from affected organs in the injected mice group while direct drug infusion to tumor cell effectively kill the cancer cell and used up mostly, facilitated from sustained release, in mice group treated with patch. This is worth mentioning here that the greatest advantage of using patch of CDgPU-L copolymers is its delivery pattern of drug in best possible sustained manner continuously for 48 h as evident from in vitro drug release profile against burst release observed in pure drug and drug loaded CD due to their powdery form and less interactive system as opposed to strongly interactive system prevail in graft copolymer drug system. Therefore, grafting plays an important role in modification of properties of polymer as per the requirement for various applications by suitably maintaining the architecture of the molecule along with hydrophilic-hydrophobic balance and proven to be effective biomaterials for the treatment of melanoma cancer in future through controlled drug delivery.

3.6 Conclusion

According to previously reported polyurethane synthesis methods here in this work efficacy of grafting polyurethane on CD for applications like control drug delivery, cell killing and in vivo animal studies on melanoma tumor is visualized. Application of PU for grafting on CD yielded copolymers with high and low graft density resulting in coverage of cyclodextrin ring which led to sustained drug release from copolymers against burst release

in pure polymer. Graft copolymers with varying degree of substitution *i.e.* graft densities have been prepared whose structures are confirmed through proton NMR, Fourier transform infrared spectra and Ultra violet Visible studies. Grafting of PU alter the hydrophilicity of CD ring as understood using contact angle measurement. Thermal stability of the copolymer enhances by more than 50 ^{o}C while the ductility rises enormously (more than 1000%) with increasing graft density. Intermolecular interaction through hydrogen bonding is prominent in high graft density copolymer against intramolecular interaction is the prime phenomenon causing smaller blob size (~0.5 nm) as measured from small angle neutron scattering experiment.

Sustained drug delivery is achieved using graft copolymer as compared to burst release noticed in CD or pure drug. Good interaction between drug and graft copolymer, as evident from the shift in melting peak, lowering of heat of fusion and blue shift in UV-Vis absorption peak, is responsible for the slow and steady release of drug from the matrix copolymer. Graft copolymers are biocompatible as determined from the cellular studies [155]. Drug embedded in graft copolymer exhibits very high HeLa cell mortality (80%) as opposed to pure drug or drug embedded in CD (meagre 20% in 5 days of culture). Animal model experiments show significant suppression of tumor volume using drug embedded in graft copolymer as compared to control or drug embedded in CD arising from sustained release of drug from the dermal patch. Histopathological analysis of organs after treatment revealed that liver and kidney were normal in mice treated with graft patch against severe damage in mice treated with pure drug. Biochemical parameters also support the normal blood parameters in mice group treated with the drug embedded in graft copolymer while higher values of these parameters in control/pure drug treated group indicate dysfunction of

liver and renal disorder. Conventional injection chemotherapy has also been performed to compare the relative tumor healing and the dermal patch is found to be superior than the injection in terms of side effect, arises from the burst and spreading out of drug from the tumor site. Both *in vitro* and *in vivo* studies confirm the efficacy of the sustained release of drug from the graft copolymer without any side effect and helps in understanding the future novel drug delivery vehicle for the treatment of melanoma.