Chapter-3

Chapter 3

Chemical Modification of Poly(vinyl chloride) for Blood and Cellular Biocompatibility

3.1 Introduction

Poly(vinyl chloride) (PVC), synthetic polymeric materials have been widely used in biomedical applications including clinical analysis of salt, blood storage, catheters etc. [Gilson et al. (2000)]. Its mechanical properties and excellent capability to acquire desired functional group makes it a choice of research in the polymer field right from the early 19th century [Braun (2004)]. Over the past few decades, several reports for improvement of biocompatibility of PVC have been found in literatures [Zhao et al. (2008)]. In addition, several studies have investigated the relationship between the degree of hydrophobicity, surface charge and cellular adhesion to examine their influence on the attachment and spreading of cells onto the surface of material that finally helps concluding the success or failure of a biomaterial [Speranza et al (2004); Herrero et al. (2006) and Theo et al. (2004)].

In view of the given structure-property relationship of a biomaterial, modification of PVC is motivated for alteration in its properties and hence developing its biocompatible forms. The modification of PVC leads to change in surface properties such as, surface chemistry, surface energy, surface topography, etc. that could be critical for assessing the biocompatibility. Therefore, such modifications of polymer play crucial role in revealing their antimicrobial efficacy and thus their selection for consideration in medical applications [Kameda et al. (2011); Zhengbao et al. (2009)]. One of the important applications of biocompatibility is blood compatibility of PVC that can be

improved by adsorption of biological molecules such as heparin [Balakrishnan et al. (2005)], PEG [James et al. (2003)], fibronectin [Jian-Lin et al. (1997)] and self-assembled hemocompatible coating on its surface. In addition, various reported methods [Moulay et al. (2010)], showing surface modification by specific chemical groups [Joseph et al. (1939)] reveal an enhancement in hydrophilicity of the PVC surface that is vital in governing their biocompatibility.

The main principle behind the modification of PVC is nucleophilic substitution reaction that provides an opportunity for steady replacement of chlorine atoms through desired atoms or groups without any side reactions, resulting in modification of the surface charges that dominate at the interface between the biomaterial surface and biological environments [Khorasani et al. (2007)]. In the present work, a simple process to formulate PVC resin with thiosulphate, thiourea and sulphite has been demonstrated.
To improve PVC, it has been chemically modified with thiosulphate, thiourea and sulphite. To examine the characteristics of the newly synthesized polymers, we have examined thermal stability, surface morphologies, hydrophilicity and antibacterial activity. Finally, biocompatibility of the modified polymers has been assessed through hemolysis and thrombosis tests as well as cell-based assays.

3.2 Results and discussion

To identify the characteristics of newly synthesized polymers, we have examined the thermal stability, surface morphologies, hydrophilicity and antibacterial activity. Finally, biocompatibility of the modified polymers has been assessed through hemolysis and thrombosis tests as well as cell-based assays.

3.2.1 Nuclear magnetic resonance spectroscopy

(1) **PVC-** ^{1}H NMR (in DMSO): δ = 1.30 (s, 3H), δ = 3.34 (d, 2H), δ = 4.46 (s, H) [indicative of pure PVC]
(2) **PVC-TS-** ^{1}H NMR (in $CDCl_3$) : δ = 1.01 (t, 3H), δ = 1.65, 1.47 (d, H_2O present in $CDCl_3$), δ = 2.32 (symmetric), 2.09(anti- symmetric) (d, 2H, CH_2CCl), δ =

3.75, 3.28 (s, 1H, HCS), δ = 4.29, 4.59 (due to presence of hydrogen bonding), δ = 4.46 (s, H-C-Cl), δ = 7.25 (solvent) [indicative of PVC modified with $S_2O_3^{2-}$]

(3) **PVC-TU-** ^{1}H NMR (in DMSO) : δ = 0.96 (t, 3H, CH3), δ = 1.35, 1.58 (m, CH_2), δ = 3.21 (m, 2H, HC-SNH_2) δ = 4.46 (s, H-C-Cl), δ = 8.22 (s, NH_2) [indicative of PVC modified with $CSNH_2$]

(4) **PVC-S-** ^{1}H NMR (in $CDCl_3$) : δ = 1.01 (t, 3H), δ = 1.25 (m, CH_2CH_3), δ = 1.58 (s, H_2O present in $CDCl_3$), δ = 2.32 (symmetric), 2.09(anti- symmetric) (d, 2H, CH_2CCl), δ = 3.97 (s, 1H, HCS), δ = 4.30, 4.58 (due to presence of hydrogen bonding), δ = 4.459 (s, H-C-Cl), δ = 7.25 (solvent) [indicative of PVC modified with SO_3^{2-}]

The signal of the CH-Cl protons at 4.46 ppm decreases with conversion, while new one at 3.75, 3.28 and 3.97 ppm is formed due to CH-S protons in PVC-TS and PVC-S respectively.

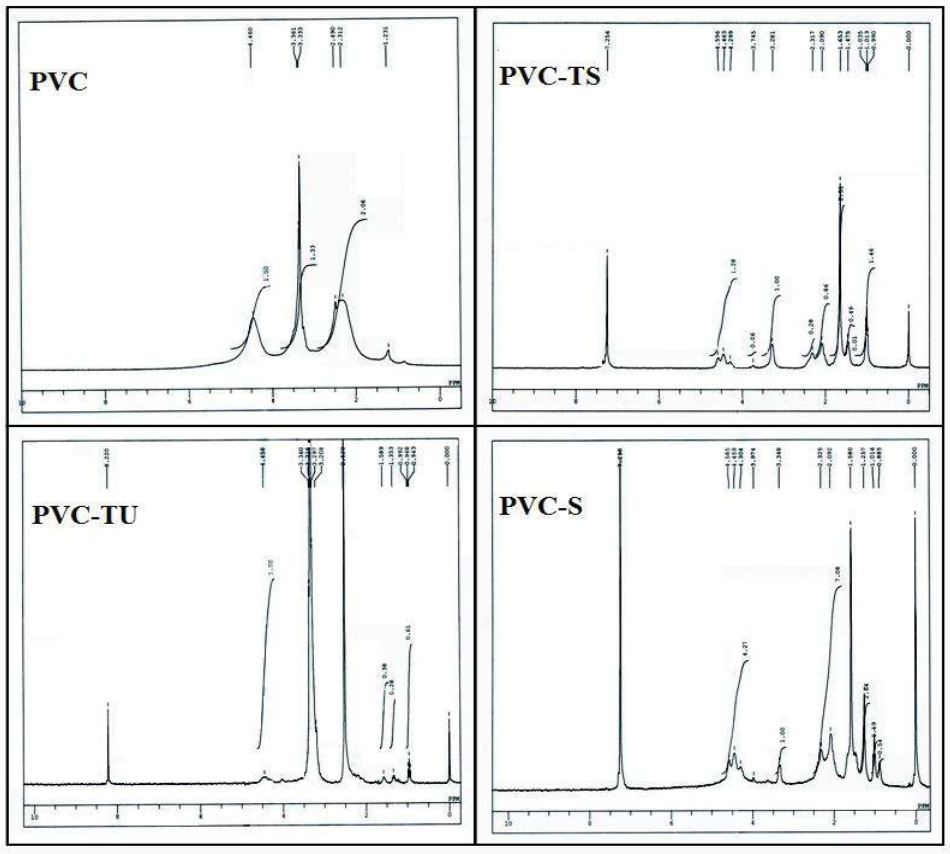

Figure-3.1: ^{1}H-NMR spectra of PVC and functionalized PVC with thiosulphate (PVC-TS), thiourea (PVC-TU) and sulphite (PVC-S).

3.2.2 Fourier Transform Infrared spectroscopy

Figure 3.2 shows FTIR spectra of polymeric PVC and functionalized PVC materials. A number of characteristics peaks can be observed: stretching of C-H of CHCl at 3200-2700 cm^{-1}, wagging of methylene groups at 1430 cm^{-1}, stretching of C-H of CHCl at 1258 cm^{-1}, 1065 cm^{-1} for C-C stretching, 966 cm^{-1} for rocking vibration of CH_2 and, 614 and 695 cm^{-1} represent vibration stretching of C-Cl bonds of syndiotactic and isotactic structures of PVC. Similar structure has been reported in the previous literatures [Seeponkai et al. (2013)].

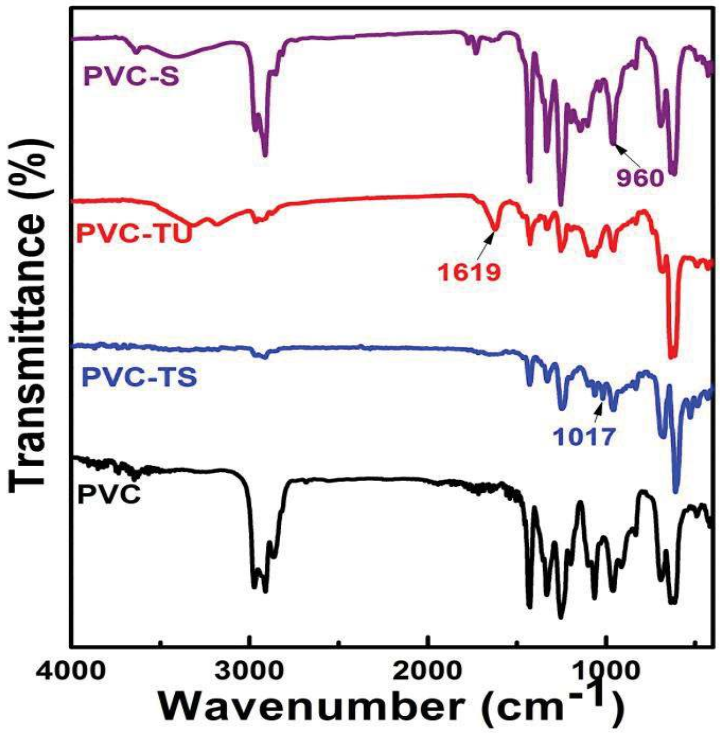

Figure 3.2: FTIR spectra of pure and functionalized forms of PVC.

The structure of modified polymers was established on the basis of replacement of chlorine atom in the polymer chain. The presence of nucleophile was confirmed by the FTIR and UV spectroscopy. Fig. 3.2 shows the IR spectra of pure and modified forms of PVC; thiosulphate ($S_2O_3^{-2}$) and sulphite (SO_3^{-2}) groups show the $S_2O_3^{-2}$ stretching at 1017

cm^{-1} and 960 cm^{-1} respectively, strong stretching of C-S at 690 $cm^{-1}$with weak stretching of C-S-S-C [Lakshmi et al. (2002)] at 540 cm^{-1}, (PVC-Thiourea) NH stretching observed at 3315 cm^{-1} and 3180 cm^{-1}, 1619 cm^{-1} may be due to N-H bending, while at 1425 cm^{-1} for N-C-N stretching in thiourea substituted PVC and (PVC-Sulphite) having C-OH group at 3420 cm^{-1}. Thus, the obtained data indicates that PVC was successfully modified with different functional groups by nucleophilic substitution reaction. Kameda *et al.* [Kameda et al. (2009)] have shown substitution of the chlorine ion by I^-, SCN^-. OH^-, N_3^- and pthalamide anions in PVC resins using a nucleophile solution and thus developed various forms of polymers with enhanced conductive property and substantial antibacterial activity.

3.2.3 UV-visible spectroscopy

The absorbance of UV-Vis light by polymeric material is mainly attributed to electron transitions among the σ, π and *n* energy levels from the ground state to higher energy states. The UV-Vis spectra in wavelength range of 200-400 nm of PVC and its derivatives have been shown in Figure 3.3. One absorbance peak was observed in PVC near 206 nm is due to *n*-π* transition. Another absorbance peak, observed in PVC-TS samples at 209-249 nm, is credited to π-π* transition due to conjugation. As can be seen, there are sharp absorption peaks at 218 nm for thiosulphate, 249 nm for thiourea and 209 nm for sulphite. Khan et al. (2012) have used sodium thiosulphate and sodium sulphite for the identification of polysulfide and oxidized sulphur species together and observed the similar results for thiosulphate and sulphite anion. In addition, Mushtari et al. (2009) have found such transition peak due to C=S chromophore in the derivatives of pyridylthiourea. Similarly, Madhurambal et al. (2010) have observed comparable results while analyzing urea and thiourea with urea-thiourea-zinc chloride crystal. The peak in favour of *π*-π** has shown red shift in modified PVC with respect to pure PVC due to presence of different functional groups.

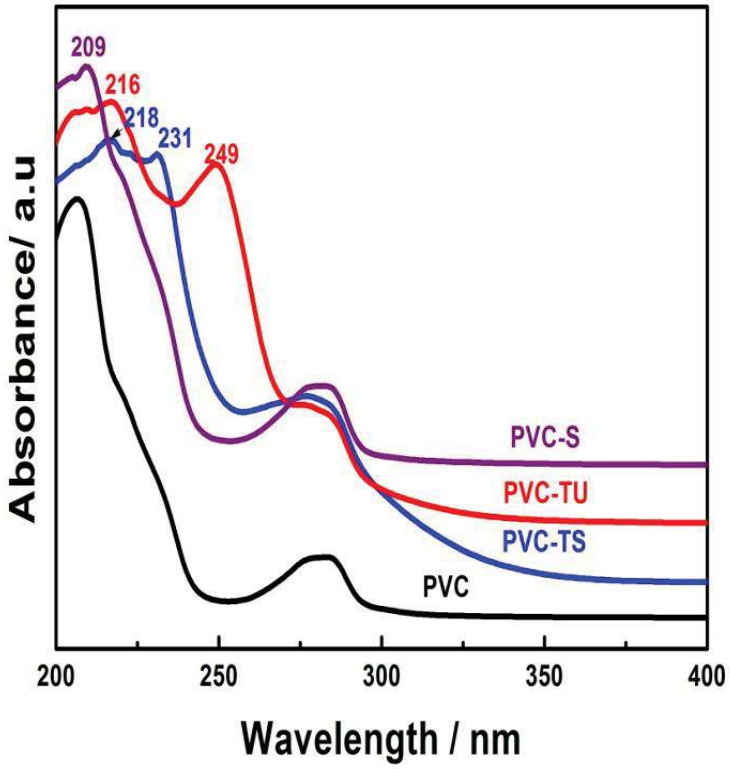

Figure 3.3: UV-Vis spectra of PVC and its derivatives.

3.2.4 Thermal gravimetric analysis

TGA analysis of pure PVC and functionalized PVC has been shown in Figure 3.4. Two transition steps can be observed from the thermogram of pure PVC of which the first step corresponds to the weight loss caused by the dehydrochlorination of PVC that begins at a temperature of 240^{o}C, while the second transition step represents the total weight loss resulted from the degradation of the dehydrochlorinated residues [Seeponkai et al. (2013)]. Whereas in case of PVC-TS, PVC-TU and PVC-S, the first transition step starts at the onset of 200^{o}C, 218.7^{o}C and 190^{o}C, respectively, while the second transitions step of all functionalized PVC is similar to that of pure PVC. The thermal degradation temperature of functionalized PVC shifts slightly to a lower temperature in comparison to pure PVC. The outcome clearly shows significant differences in the range of thermal degradation temperatures of pure and functionalized PVC resins. This shows that

existence of functional groups in the polymer chain significantly promotes the degradation of functionalized PVC (i.e. lowers the thermal stability).

However, there have been contrasting reports regarding the thermal stability of PVC upon chemical modification. A study indicates an increment of around 50°C in degradation temperature when PVC is incorporated with polyethylene glycol [James et al. (2003)]. Thermal stability is generally expected to increase upon chemical crosslinking in the polymer. In some cases, however, literature reveals that it may also decrease [Fiaz et al. (1998)].

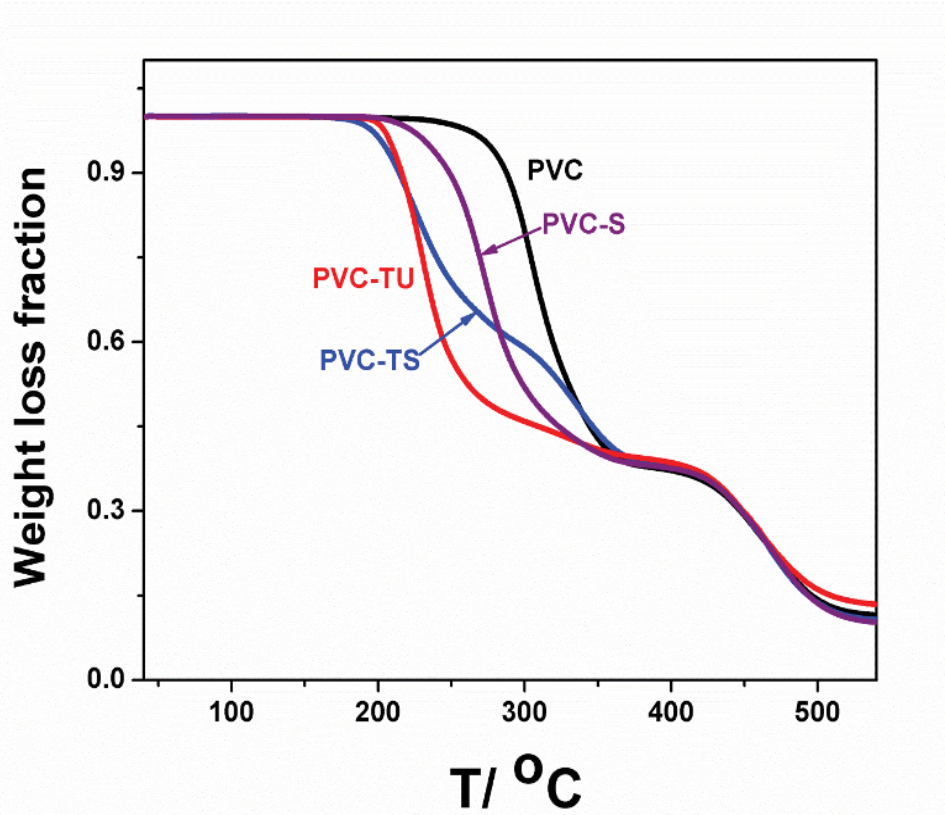

Figure 3.4: Thermogram of pure and functionalized PVC analyzed in a nitrogen atmosphere.

3.2.5 Contact angle

Figure 3.5 shows the materials' relative hydrophilicity and hydrophobicity evaluated by the contact angle measurements of synthesized polymers in contact with water. The

influence on wettability property of the materials upon chemical modification was examined and represented in Figure 3.5. The chemical modification of PVC results in a significant decrease in the contact angles, indicating that the modified polymers are more hydrophilic, which is an important factor in governing the wettability of a biomaterial, as it promotes cell growth and proliferation and thereby influences the biocompatibility property of a biomaterial. Results show that the average values of water contact angles of pure PVC, PVC-TS, PVC-TU and PVC-S are around 82º, 65º, 55º and 60º respectively, within the accuracy level [Xiaoxian et. al (2015)] of ±1º. Previously, James et al. (2003) showed similar improvement in the hydrophilic property of plasticized PVC by modifying its surface with thiocynate. Furthermore, they found that the hydrophilic property of their modified material was not supportive for bacterial adhesion, typically observed for *S. epidermidis* and *S. aureus* [James et al. (2003)]. Similarly, Lakshmi et al. (2002) showed an enhancement in the degree of hydrophobicity of the plasticized PVC upon surface modification with thiosulphate and found that the modified PVC exhibited significantly greater hemolytic activity as well as lower cellular adhesion with fibroblast cells.

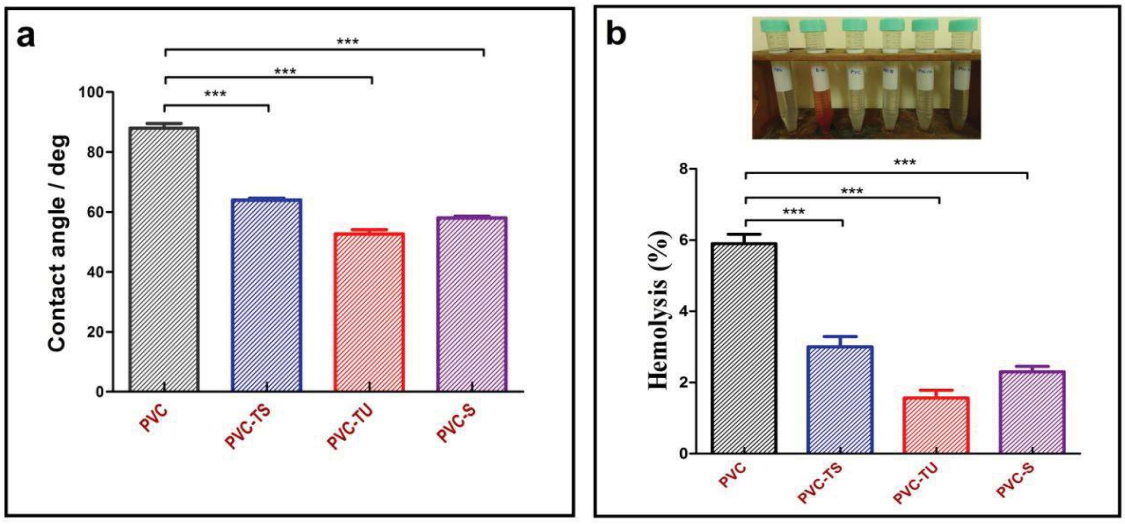

Figure 3.5: (a) Contact angle measurements of pure PVC and functionalized PVC resins and (b) Hemolysis percentage of pure PVC and functionalized PVC polymers.

3.2.6 Scanning Electron Microscopy

Figure 3.6 shows SEM images of PVC residues modified with thiosulphate, thiourea and sulphite. No significant difference in the surface morphology of pure and modified PVC particles was observed in SEM. Irregular and uneven particle morphologies were prominently observed in all cases. Whereas, a notable difference in the wettablity property of pure and synthesized PVC resins was revealed by contact angle measurements of polymer films. The modifed PVC surface was found to be more hydrophilic as demonstrated by a significant decrease in their water contact angles. Similarly, their surface charge varies quite distinctly though the surface morphology of pure PVC particles appears similar to that of treated PVC particles (Fig. 3.6). The modified PVC particles show highly charged surface due to the presence of ionic groups. Thus, the results indicate that the nucleophilic substitution of ionomers viz. thiosulphate, thiourea and sulphate does not alter the morphology of PVC surface, yet significantly affects the wettability of PVC resins.

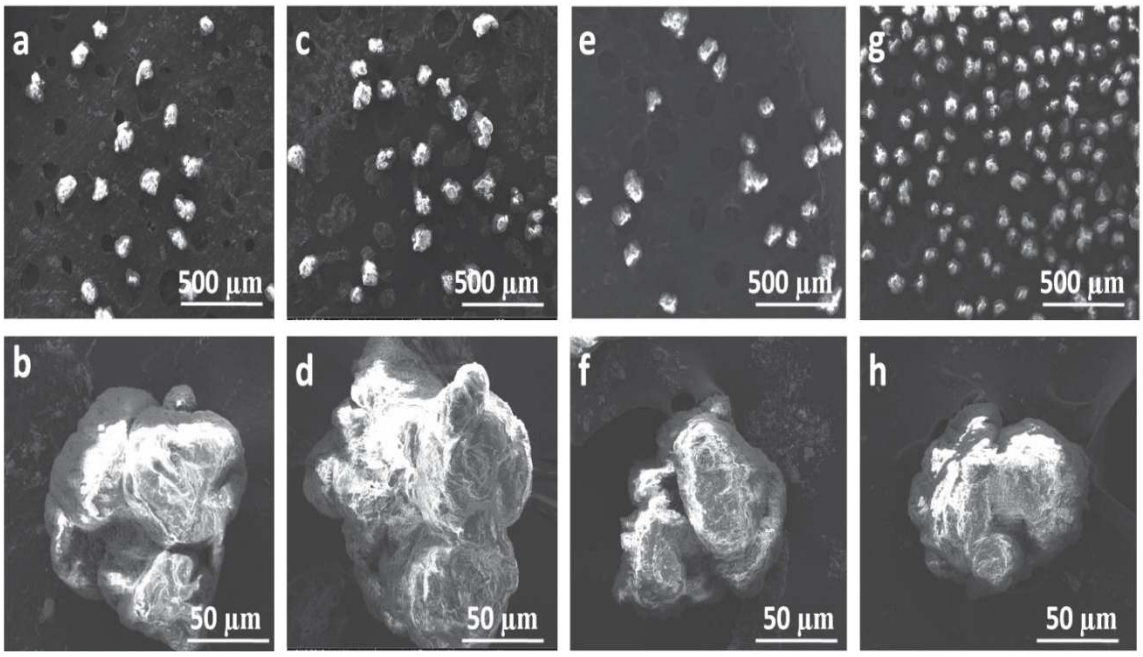

Figure 3.6: Scanning Electron Micrographs of PVC and the derivatives of PVC resin after the chemical modification. (a) & (b) PVC, (c) &(d) PVC-TS, (e) & (f) PVC-TU and (g) & (h) PVC-S.

3.2.7 Antibacterial activity

Bacterial adhesion is a complex process whose numerous aspects till date have not been well understood due to the involvement of a number of physicochemical factors in this process [Pavithra et. al (2008)]. While measurement of bacterial adhesion is important itself, it alternatively serves as a basis to characterize the antibacterial property of biomaterials [Shim et. al (1996)]. The degree of antibacterial activity based on bacterial adhesion on polymeric samples for 24 h is presented in Figure 3.7. Although the bacterial adhesion is reportedly a dynamic process, the observation was performed after 24 h incubation for a better assessment of the adhesion formation. In all cases the data revealed no decrease in the colonies of the plated bacteria that were pre-adhered to the surface of pure and modified samples; implying the inefficiency of the modifications in reducing the adherence of *E. coli* onto the polymer surface.

3.2.8 Hemolysis

Hemolysis phenomenon of blood is a major concern associated with bio-incompatibility [Gupta et. al (2013)] Hemolysis occurs when red blood cells come in contact with water and it is an important parameter to ensure biocompatibility of the material. In the present study the data showed that the recorded level of hemolysisis is less than 5% in all the cases [Autin (1975)]; suggesting that the modified forms of PVC are advanced biomaterials and could be used as alternatives to pure form of PVC. However, attempt is in progress to further improve these polymeric materials.

3.2.9 Thrombogenicity evaluation

The weight of blood clots obtained after incubation of blood with PVC, PVC-TS, PVC-TU and PVC-S for 30 min was 1.9, 1.3, 1.6 and 1.1 mg, respectively. These results are consistent with the previous studies. Reported literature suggests that the surface properties play a vital function at a molecular level in governing surface-induced hemolysis [Ishihara et. al (2000)]. Notably, hydrophilic nature of the material directly corresponds to their improved biocompatibility. In addition, several studies suggested that a biomaterial with the positively charged surface promotes thrombogenesis when

exposed to blood, while negative charged biomaterials tend to suppress the thrombogenesis process [Black (1999)], most likely due to the fact that blood cells and platelets have net negative charge on their surface.

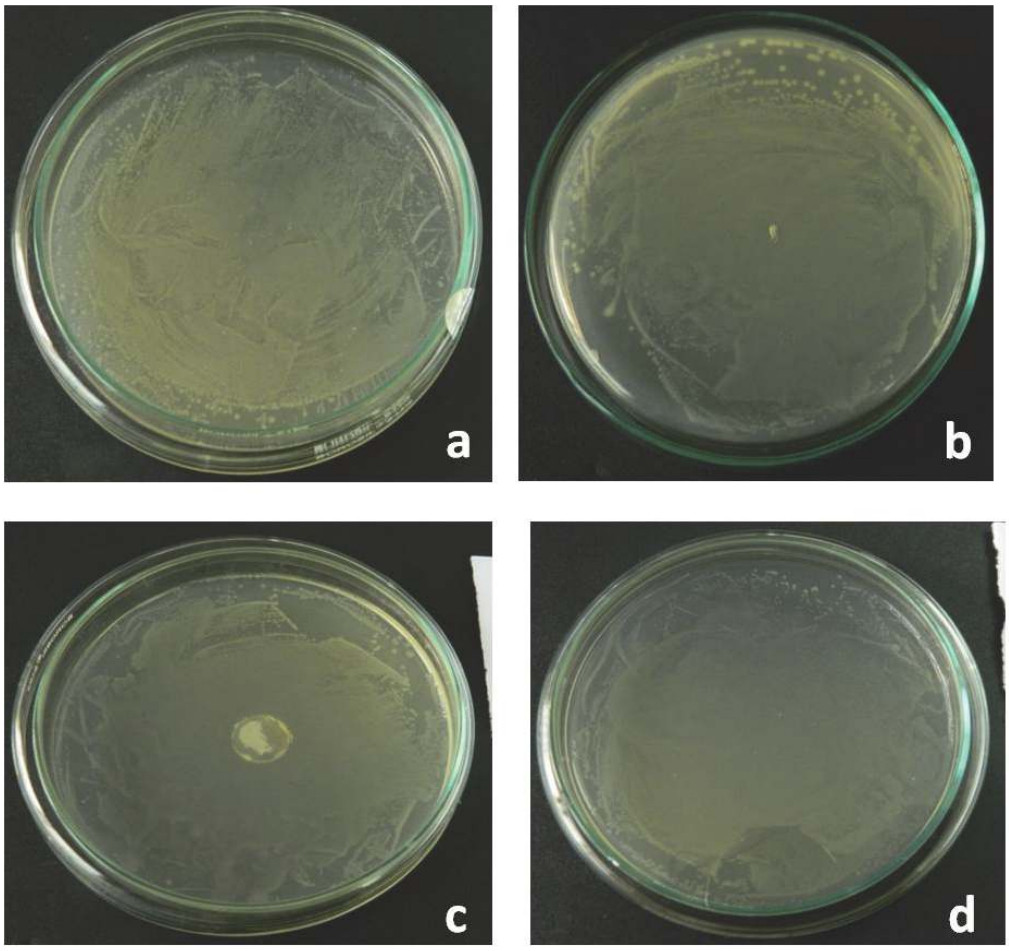

Figure 3.7: Antibacterial activity of PVC and its functionalized polymer; colonies of E. coli grown on (a) PVC, (b) PVC-TS, (c) PVC-TU and (d) PVC-S.

3.2.10 Cell adhesion

All forms of polymers supported cellular adhesion under the standard conditions. Figure 3.8 shows the percentage of mMSCs adhered to PVC, PVC-TS, PVC-TU and PVC-S polymers after 4 h. Polystyrene tissue cultured Petri dish (without sample) used as a control in all cases. The total set of modified polymers shows significantly higher level of

adhesion percentage compared to the pure form of PVC. The level of cellular adhesion was found notably reduced on PVC-TS surfaces compared to other modified polymers. There was no significant difference observed between PVC-TU and PVC-S as both showed relatively similar range of cellular adhesion on their surface. Previous studies suggested that the functional groups present on the surface of a biomaterial directly influences biocompatibility. Curran et. al (2006) has investigated the importance of functional groups in governing cellular adhesion using human mesenchymal stem cells. They have demonstrated the adhesion behavior of cells with methyl, amino, silane, hydroxyl and carboxyl groups and shown that all surfaces maintained viable cellular adhesion throughout the test period.

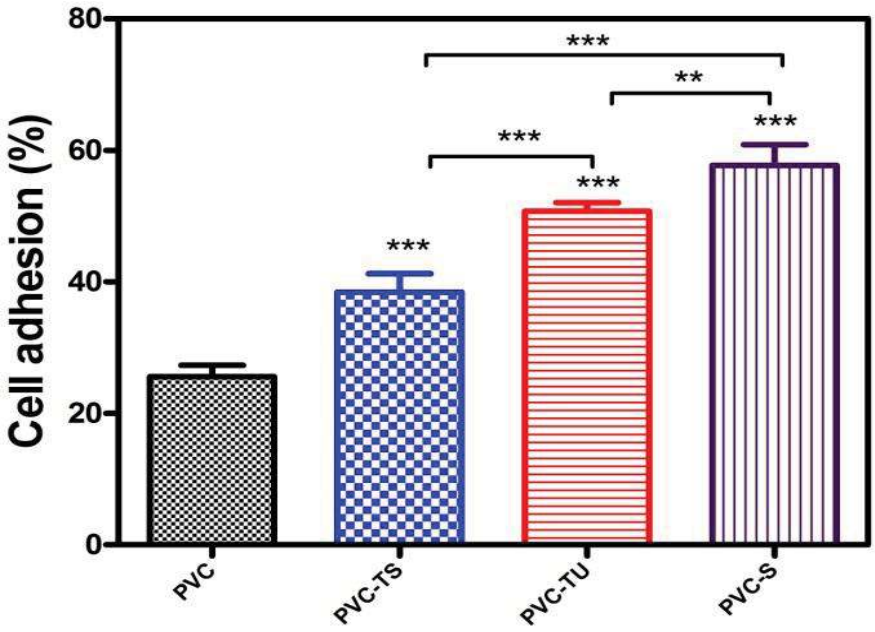

Figure 3.8: Biocompatibility evolution of PVC and its derivatives. (a) The percentage value of mesenchymal stem cell adhesion on PVC and its functionalized forms was evaluated using crystal violet. The absorption values were taken at the wavelength of 544nm.

*P < 0.05
** P <0.01
*** P < 0.001

3.2.11 Cell viability

To determine effects of the functional polymers on metabolic activity, the MTT test was performed. Cytotoxicity of polymeric materials after their incubation with cells for 1, 3 and 5 day was observed in a culture medium. The cytotoxicity was measured by determining the cellular viability using a MTT assay. Figure 3.9 represents the plot for the viability percentage of mMSCs and shows significantly lower levels of cytotoxicity in case of functionalized polymeric materials. Viability of the cells seeded on a bare tissue culture grade polystyrene petri dish was considered as a control**.**

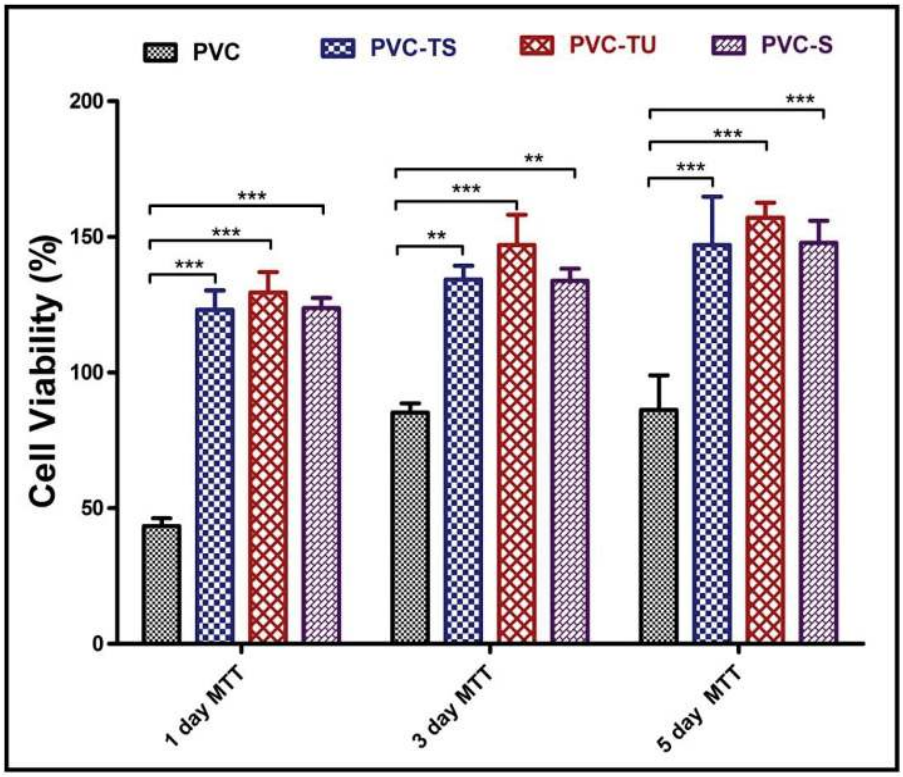

Figure 3.9: Cell viability of mouse mesenchymal stem cell seeded on PVC, PVC-TS, PVC-TU and PVC-S surface. Cells were plated directly on the polymeric biomaterial surface and cultured for 1, 3 and 5 days in a growth medium.

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

The cell viability was found to be ~ 43% for PVC after 1 day of culture while it increased significantly by another 77% ($P \leq 0.001$), 86% ($P \leq 0.001$), 80% ($P \leq 0.001$) for PVC-TS, PVC-TU, and PVC-S, respectively. Similarly, after 3 days of culture, the viability was noted to be around 42% in PVC and increased further by 49% ($P \leq 0.01$), 62% ($P \leq 0.001$), 49% ($P \leq 0.01$) for PVC-TS, PVC-TU, and PVC-S, respectively.

3.2.12 Nuclear Staining

Figure 3.10 shows the nuclei of adhered mesenchymal stem cells on PVC and functionalized PVC.

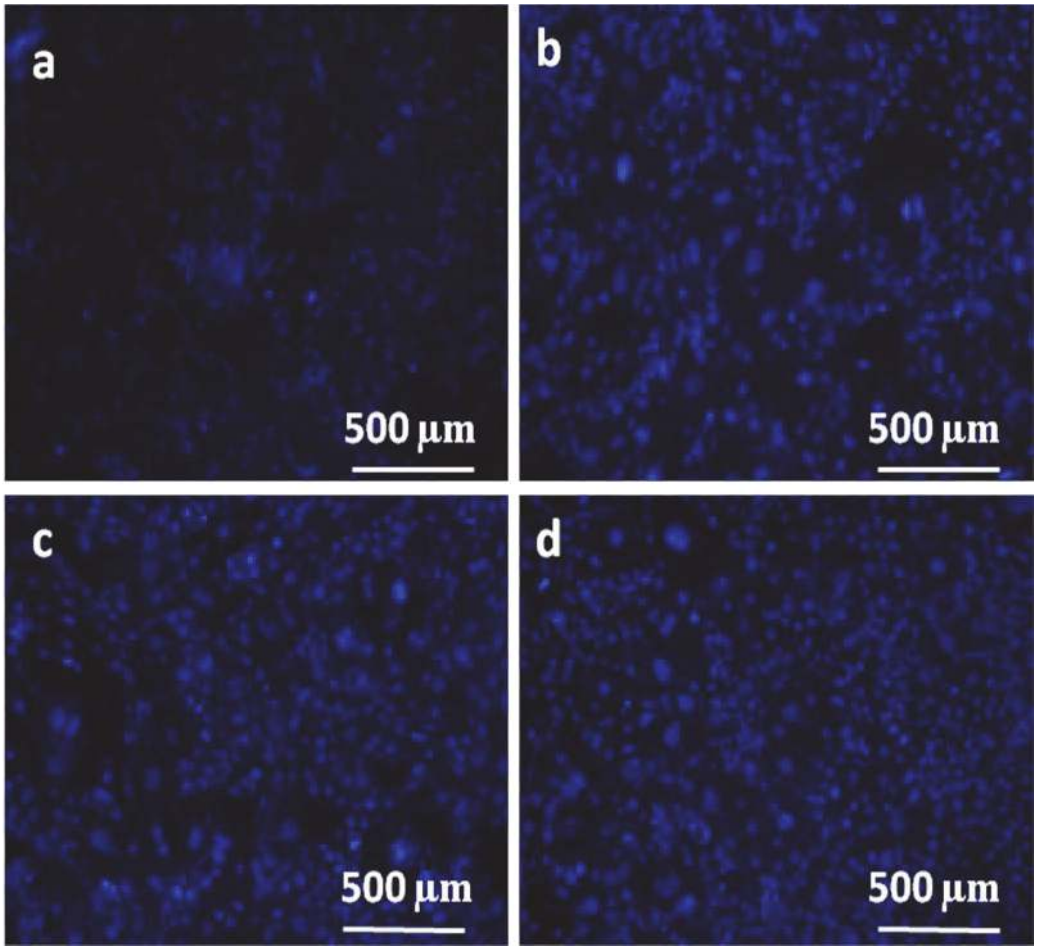

Figure 3.10: Nuclear morphology of mMSC cells grown on different polymeric surfaces for 24 h. Cells were cultured in direct contact with various samples and analyzed with a fluorescence microscopy. (a) PVC; (b) PVC-TS; (c) PVC-TU; (d) PVC-S.

Nuclear staining indicates that the cells adhered on modified forms of PVC were significantly higher in comparison to that of control PVC. Microscopic images further reveal that pure PVC does not support cellular adhesion at all while PVC-TS, PVS-TU and PVC-S assist adherence of cells to a significant extent compared to the pure material. Thus, these results suggest that modification of the PVC resins with different functional groups enhances their biocompatibility properties.

Also, similar trend was observed following 5 days of culture, ~1% for PVC while it increased by another 61% ($P \leq 0.001$), 71% ($P \leq 0.001$), 62% ($P \leq 0.001$) for PVC-TS, PVC-TU, and PVC-S, respectively. In summary, the cell viability was found to be significantly higher in case of functionalized PVC polymers in comparison to its pure form.

3.3 Summary

This work demonstrates the influence of different functional groups on the characteristics of PVC surface and the resulting biocompatibility properties. For this purpose, functionalized forms of PVC using thiosulphate, thiourea and sulphate have been fabricated by nucleophilic substitution reaction using phase transfer catalyst. The outcome revealed that functionalized polymers are hydrophilic in nature, show reduced hemolytic activity, and support bacterial and cellular adhesion significantly.