# Chapter 6 BIOLOGICAL EVALUATION OF MODIFIED METALLOCENE POLYOLEFIN

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6.1 Introduction

The term biological performance is related to the interaction between material and the living system. It has two important divisions i) host response and ii) material response. The traditional approach has been to define biological performance in terms of biocompatibility (host response). The issue of biocompatibility rises from recognition of the profound differences between living tissues and nonliving materials. The label “biocompatible” suggests that the material described displays universally “good” or harmonious behaviour in contact with the tissue and body fluids. Biocompatibility refers essentially to the effect of the material on the biological system. Since there is a broad range of materials available and only a very small percentage has been used in the biological environments, there has been a continual need for a quick screening method that can be used in vitro- outside the body. These can be divided into two classes

i) Cytotoxicity tests methods

ii) Blood contact methods (for blood contact applications)

With each of these classes there is a wide variety of tests and variations of test methods. Some variations are traditional for particular applications, while others are the practice of a particular laboratory.

The need of finding acceptable material for use in contact with the blood is of great important to the present day clinical practice. The difficulty in finding acceptable materials reflects the complex nature of blood-material interactions, which are influenced by properties of the material.

Critical biocompatibility evaluation is an essential step in the development of any material intended for biomedical applications. The performance and the biological response of biomaterials must be evaluated to determine whether their compatibility and functionality are suitable for application in physiological systems. This implies the utilization of standard practices, which
recommend generic biological test methods for materials and devices according to end-use applications. These test protocols are intended to be applied to materials and medical devices for human use and recommend sufficient biological testing to establish a reasonable level of confidence concerning the response to a given material or device to a living organism, as well as guidance in selecting the proper procedures to be carried out for the screening of new or modified materials.

Biocompatibility evaluation of medical devices involves testing either the material itself or an extract from it, or both, depending on the nature of the end-use application. For the convenience of assessment, the tests are categorized into (1) in vitro screening tests, followed by (2) in vivo toxicological/biocompatibility tests. The screening tests check the preliminary safety with respect to cells/blood and are performed regardless of the final use of the materials. In vitro cell culture cytotoxicity evaluation on established cell lines is the common screening test employed to assess the acute toxicity effects of material under study. A material passes this test only if acute toxicity effects are not recorded; further tests are then done on the material to assess the toxicological/biological response by making use of suitable animal models. In vitro test methods are usually quicker and less costly than in vivo methods and do not require the use of animals.

The International Organization for Standardization (ISO) recommends a battery of biocompatibility studies to be carried out on all medical devices in accordance with their end-use applications. For example, for a material intended for blood or blood component storage applications, the following battery of tests have to be done based on the guidelines/protocols prepared by ISO-10993 (1):

1. Cell culture cytotoxicity
2. Cell adhesion studies
3. Haemolysis assay
4. Clotting time test

The biological performance of the modified metallocene polyolefin with potential future use as blood and blood component storage containers is analysed in this chapter by following standard practices for generic biological test methods for materials and devices. Mechanical and permeability property evaluation of the mPO-EVA blends (chapter 5) showed that the blends, prepared from mPO and EVA12, were significantly superior to the other compositions studied. The most suitable composition, namely AE1225 was selected for biological evaluation. Biological evaluation studies such as cell culture cytotoxicity, cell adhesion, haemolysis and clotting time were carried out to gauge the biological performance of these blends.

6.2 Experimental

In vitro cell culture cytotoxicity of AE1225 and AE1255, the two modified samples of mPO were evaluated by direct contact method and test on extract method as per ISO 10993-5 (1999) using L929 mammalian fibroblast cell lines. The blood compatibility of the modified sample (AE1225) was evaluated by platelet and leukocytes adhesion studies, clotting time tests and haemolysis assay. In the clotting time test the kinetics of thrombus formation were determined using fresh human blood according to Xianghuai et al.(2).
6.3 Results and Discussion

6.3.1 In Vitro Cell Culture Cytotoxicity

Cytotoxicity is the ability of a material or substance to produce a toxic or cellular effect with a deviation from normal morphology and functionality (2). This testing is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The cytotoxicity tests essentially consist of the assessment of cell morphology and viability when the cells come in contact with either the material or the extract of the material. The cells lysed or injured by the material or the extract of the material are examined. Generally, established mammalian cell line is used for the study. The L929 (mouse fibroblasts) have become the most commonly used cell line because they are easily cultured and scored. They also represent a cell line capable of being perpetually subcultured and have been shown to be among the preferred cell types for use in biocompatibility test procedures in vitro (3). The international standards compiled as ISO 10993, and the FDA blue book memorandum (#G95-1) that is based on ISO10993-1, address the critical issue of ensuring device biocompatibility by identifying several types of tests for use in selecting device materials. Required for all types of devices, cellular toxicity testing is covered in ISO 10993-5: “Tests for Cytotoxicity—In Vitro Methods” (4) This standard presents a number of test methods designed to evaluate the acute adverse biological effects of extractables from medical device materials.

The response of AE1225 and AE1250 to a culture of L929 cells is shown in figure 6.1 (a & b).
It can be seen from the figures that the typical spindle shape morphology of L929 was retained even after 24 h of contact with AE1225 and AE1250. Similar results were obtained for the test performed on the extract of the blend materials (Figure 6.1c&d). The results from the in vitro cell culture cytotoxicity showed that both AE1225 and AE1250 were non-cytotoxic to L929 cell line.

Figure 6.1  L929 cells incubated with (a) AE1272, (b) AE1255, (c) extract from AE1272 and (d) extract from AE1255 over 24 h.

Figure 6.2 (a & b) shows the shape of the cells in a positive and negative cytotoxicity control samples respectively. A positive cytotoxicity test result can be taken as an early warning sign
that a material contains one or more extractable substances that can be of clinical importance. In such cases, further investigation is required to determine the utility of the material.

Figure 6.2 L929 cells incubated with (a) positive cytotoxicity control sample and (b) negative cytotoxicity control sample over 24 hours.
Chapter 6

6.3.2 Clotting Time

The initial event that occurs when blood contacts a foreign surface is the adsorption of plasma proteins, followed by a complex series of reactions that include the activation of blood-clotting enzymes and adhesion and activation of blood platelets and leukocytes, desorption and or further adsorption of proteins (5,6). When an abnormal situation comes, the cells in the blood trigger the blood-clotting cascade. The initiation of clotting cascade mainly depends upon the situation and foreign surface. Material mediated clotting initiation depends on the nature of the surface properties of the material. Lee and Neville reported the clotting times for various untreated plastics and indicated different clotting times for different base polymers (7). Slow clotting initiation means clotting time is more and fast clotting initiation indicates less clotting time. In the biocompatibility point of view a long clotting time indicates the material is suitable for blood contact applications.

In the whole blood clotting time test method a specific quantity (0.1ml) of blood was placed on the surface of the material. After a predetermined time the specimen was transferred into a beaker containing 50ml of water. The red blood cells that has not been trapped in the thrombus were haemolysed in the water and was measured at 540 nm in a uv-visible spectrophotometer. The optical density of the solution was monitored as a function of time and was plotted as shown in the 6.3. Conventionally, the time at which the optical density decreases to 0.1 is regarded as the clotting time.

The results show that blending of EVA with mPO increased the clotting time. It further shows that the clotting time increases with increase of EVA content. The modified samples, AE1225 and AE1250 show longer clotting time than PPVC, which in turn indicates that both these modified samples are better than PPVC from the biocompatibility point of view.
6.3.3 Cell Adhesion Studies

When blood contacts with an artificial surface, a series of events are initiated: rapid adsorption of a layer of plasma proteins at the interface, platelet adhesion to the protein layer and activation of the coagulation system to form thrombin and fibrin and culminate in thrombus formation (8,9). Platelets are anucleated fragments of megakaryocytes, which are formed in the extra sinusoidal space of the bone marrow. The typical shape of resting platelets is discoid (Figure 6.4a). Platelets are the smallest corpuscular components of human blood (diameter 2-4 µm) - the physiological number varies from 150,000 to 300,000/mm³ blood. Despite their appearance on the face of it platelets are not cells, as they are not provided with a nucleus. Platelets circulate in the blood and their normal life span is 8-10 days and 1/3 of the platelet mass is transiently sequestered in the vascular pool of the spleen, exchanging freely with the systemic vascular pool. Platelets have important physiological role in maintaining vascular integrity and the arrest of bleeding by its interaction in association with coagulation factors with the component of vessel wall (10).

![Figure 6.4: Scanning electron micrographs of platelets (a) and its activated form (b)](image)

Platelets play a major role in determining the short-term thrombogenicity of artificial surfaces exposed to blood. When platelets come in contact with any artificial surface, they get activated and upon activation they undergo a shape change to a globular form with pseudopodia (Figure 6.4b). Platelet-material surface interactions are major determinant of thrombus when blood is exposed to artificial surfaces (11). Similarly, leukocytes are another type of cells found in the blood. The adhesion of these cells to the artificial surfaces also give rise to cell activation and the generation of complement and coagulation proteases, which in turn may mediate thrombus formation and inflammatory reactions (12). So in the biocompatibility evaluation of materials to be used for blood contact application, the adhesion studies of platelets and leukocytes are very much important and essential.

The cell adhesion test was carried out as per the International Standard ISO10993-4: 2002 (13). Blood from human volunteer was collected into the anticoagulant, CPD. The test materials were
placed in polystyrene culture plates and immersed in phosphate buffered saline before they were exposed to blood. To each plate 1.5ml blood was added and a 0.5ml sample was collected immediately for cell count. Three samples were tested for each material. Three empty polystyrene culture dishes were exposed with blood as reference. The count reduction was analysed by detecting the counts in initial and 75min samples using Haematology Analyzer (Cobas Minos vet, Roche, France). Total consumption from the exposed blood as the percentage reduction is calculated for each sample. The total consumption means that the number of platelets or cells adhered to the artificial surface. Less count reduction means that less number of particles is adhered on the artificial surface and is more advantageous from the biocompatibility point of view.

The percentage platelet count reduction on the sample surfaces is shown in the figure 6.5. The results indicate that the platelet count reduction is more in the case of virgin m-PO (no modification) compared to PPVC. So it may be inferred that the virgin m-PO is less biocompatible than PPVC. A low level reduction of cells from the medium can be seen when a modified material comes in contact with it. This indirectly indicates that the modification of mPO with EVA reduces adhesion of cells onto the surface and this is a very encouraging result as far as the biocompatibility is concerned.
Similarly, the leukocyte-material interaction study as shown in figure 6.6 indicated that very less number of cells is adhered to the virgin and modified samples of mPO compared to PPVC. Since further leukocytes count reduction was not possible from the virgin material from the practical point of view, the assessment of the modification was not possible.

Figure 6.5 Percentage platelet count reduction on the sample surfaces

Figure 6.6 Percentage leucocyte count reduction on the sample surfaces
For the visual assessment of the platelet and leukocytes adhesion onto the artificial surface, 1.0 ml of the blood was exposed to the materials for 30 min under agitation at 75±5 rpm using an Environ shaker thermostated at 35±2°C. The materials after lapse of the time period were rinsed thoroughly with phosphate buffered saline and

were fixed with 1% glutaraldehyde for 1 hour. They were then stained with May Grunwalds stain and viewed under light microscope to detect cell adhesion. A large number of platelets and leukocytes were seen adhered on the pPVC sample surface (figure 6.7). Adhesion of cells was very less in the case of virgin m-PO and light microscopic results indicate that the modification of m-PO improved the non-thrombogenicity of the material (figure 6.8 & figure 6.9)

### 6.3.4 Haemolysis

Red blood cells are extremely numerous in the blood stream and each has an average life expectancy of about four months. The destruction of red blood cells appears to be a major concern when plastic implants are to be in contact with flowing blood on a long-term basis. Plastic surfaces have been implicated in immediate and delayed haemolysis.
Comparison of percentage haemolysis of virgin mPO and its modified version (AE1225) was carried out with pPVC and the results are given in the figure 6.10. The results indicate that the percentage haemolysis is less for both the virgin mPO and the modified sample AE1225. It further shows that the modification mPO with EVA reduced the percentage haemolysis.

![Graph showing comparison of haemolysis percentage between pPVC, mPO, and AE1225](image)

Figure 6.10 Comparison of percentage haemolysis of virgin mPO modified sample (AE1225) and pPVC.

**6.4 Conclusion**

The biological evaluation of the blend composition AE1225 was investigated based on the guidelines/protocol prepared by ISO-10993. Results of the cytotoxicity potential evaluation revealed that no cellular degeneration or malformation occurred to the 1929 cell lines when exposed to the blend composition AE1225. The cell line P929 retained their original morphology even after being in contact with the samples either directly or by means of the extracts from the material, suggesting that the material is non-cytotoxic to the cell-lines used for the study.

The data on clotting time of the materials show that the modification of mPO with EVA increased the clotting time of the virgin polymer. On comparison of the clotting time of the blend AE1225 with PPVC the former showed a higher value indicating its potential candidature for the replacement of pPVC.

The reduced percentage reduction of the platelets and leucocytes counts in the material exposed blood samples indicated that the modification of mPO with EVA reduces cells loss from the blood, which in turn indicated less adhesion of the cells on the sample surface. This result was confirmed with the microscopic surface evaluation of the materials. Compared to pPVC the
virgin mPO and the blend AE1225 shows less adhesion of the cells which is a positive, and encouraging result in the cell adhesion study point of view.

The haemolysis test carried out gives an understanding about the material mediated blood cell lysis, especially the leucocytes. The results showed that the virgin mPO and the blend AE1225 were less haemolytic than PPVC. Also the modification of mPO with EVA reduced the haemolytic nature of the material.

The in vitro blood compatibility evaluation of the materials show that the blend AE1225 exhibits better haemocompatibility and lower cell adhesion compared to that of plasticized PVC. Therefore the blend is a promising material for blood /blood component storage

6.5 References


