

# In-vitro biological test methods to evaluate bio-resorbability

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Nowadays, biomaterials are commonly employed in medicine, odontology and biotechnology. A biomaterial must fulfil some criteria like: (i) biocompatibility; (ii) biomechanics adapted for the host tissue and (iii) degradability or un-degradability according to its future in the body.

The degradation of a material will depend on the chemical structure of the material employed (e.g. polymer, metal, ceramic); a polymer will be degraded by hydrolytic or oxidative mechanisms, whereas metals will be degraded mainly by corrosion. After implantation in the body, the material is recognized as a foreign body by the cells, mainly immune cells, whose function is to eliminate it. This starts by the adsorption of specific protein on the surface of the material which, depending on the surface material characteristics, serve as substrates for the membrane receptors located on the outer layer of the cytoplasmic membrane (mainly integrins).

In the literature, four terms are employed to describe the degradation of polymers <sup>1</sup>:

- (i) **Biodegradation**. A biomaterial is described as *biodegradable* when it is degraded into macromolecules, but macromolecules stay in the body and can migrate. For instance, it is the macromolecules of Ultra High Molecular Weight PolyEthylene (UHMWPE) generated from joint prosthesis.
- (ii) **Bioresorption**. A material is resorbable if it can be entirely bulk degraded *in vivo*. It is eliminated from the body into low molecular-weight molecules. The most contributing factor for resorbability is the time required for the total elimination of the material; a bioresorbable polymer such as polylactic acid will be eliminated much faster. However, surprisingly metals could be classified as bioresorbable as corrosion could lead to the total elimination of the material, but the time required is much longer than other resorbable polymers.
- (iii) **Bioerosion**. This term refers to materials that first are degraded on surface and then resorbed *in vivo*.

- (iv) **Bioabsorption**. In this case the material could dissolve in the body without modification of their molecular weight, a process which is in contrast to bioresorption.

In this chapter we will deal with the *in vitro* (ultimately translated in *in vivo* circumstances) modes of degradation of biomaterials, with the *in vitro* methods to assess resorption of biomaterials and finally with the characterization of resorption.

## **1. METHODS OF DEGRADATION OF BIOMATERIALS**

In the *in vitro* assay or after implantation, materials are in exposed to salt-containing solutions and biomechanical stress, which can lead to the generation of particles and wear debris and activation of the immune system (**Figure 1**). Some materials can be inert in a bulk shape and biologically active when they are in a particulate form (e.g. UHMWPE).

### **1.1. Degradation of polymers**

There are two different modes of polymers degradation: (i) the hydrolytic degradation and (ii) the oxidative degradation, which leads to the generation of debris and then to the bioresorption of the materials.

#### **1.1.1. Hydrolytic degradation**

The hydrolytic degradation is the cleavage of functional groups by water reaction. This process can be catalyzed by acids, bases or enzymes. In this kind of degradation, the cleavage rate is directly proportional to the initiator rate <sup>2</sup>. The susceptibility of a polymer to hydrolytical degradation is the result of its chemical structure (presence of hydrolysable groups) and of the surrounding biological environment.

Hydrolysable polymer contains C=O group linked to another element (O, N or S). In this family, there is esters, amides, thioesters, urethanes, carbonates, imides, anhydrides (**Figure 2**). Other polymers like, ethers, nitriles, polyphosphonates, polysulfonates, sulfonamides and polycyanoacrylates could be hydrolysable under certain conditions <sup>3</sup> (**Figure 3**). The hydrolytic rate increases with the number of hydrolysable groups, low crystallinity, hydrophilicity, absence of cross-links and the surface size of the material (a porous material will be degraded faster than a non-porous, similarly a rough surface will be degraded sooner as a smooth surface). Inversely, hydrophobic materials, cross-links, high crystalline and bulk shape decrease the hydrolytic rate.

Hydrolysis can be catalyzed by ions. Usually biological and cell culture fluids contain ions like  $H^+$ ,  $OH^-$ ,  $Na^+$ ,  $Cl^-$ ,  $HCO_3^-$ ,  $H_2PO_4^{2-}$ ,  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$  and  $SO_4^{2-}$  (**Table 1**).

It has been shown that some ions like  $PO_4^{3-}$ , can accelerate the hydrolytic rate of degradation <sup>4</sup>. The ion-mediated hydrolysis can be limited only to the surface of the polymer (hydrophobic material) or it can penetrate into the deeper layer (hydrophilic material). Modifications of pH, due to increased inflammatory reactions can increase the hydrolytic rate in the immediate proximity of the biomaterial.

Enzymes, are also known to recognize some structural motifs and initiate the degradation of the polymer <sup>5,6</sup>. For this reason, synthetic polymers are more resistant to the degradation than natural polymers. However, some studies have shown that hydrolases can initiate degradation of synthetic polyesters and polyamides <sup>7-9</sup>, but it is still unclear whether certain enzymes can initiate the degradation of  $H_2C-CH_2$  motifs. Generally, enzymes exert their activity only at the surface of the materials due to their high molecular weight impairing them to penetrate into the deep layer of the material.

### **1.1.2. Oxidative degradation**

Oxidation of polymers occurs by a homo-lytic (formation of free radicals) or hetero-lytic (one of both atoms keep the pair of electron of the chemical bond). The favourite sites for oxidative degradation are those which allow the withdrawal of an atom or an ion and the stabilization of the corresponding radical or ion (**Figure 4**). The most suitable sites are carbon substituted by an aliphatic chain, carbon substituted by an aromatic cycle or by an allylic group, ethers, phenols, alcohols, aldehydes and amines.

In this kind of degradation, the free radicals generated by the host directly induce the oxidation of the polymer. It is well known now that activated phagocytes (e.g. macrophages, neutrophils) are capable of releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the close proximity of the materials <sup>10</sup>. During the first days of implantation, neutrophils, which respond to chemical mediators at the trauma site in the acute or early phase, induce a strong and transitory attack of the material surface by the release of free radicals (ROS and RNS)<sup>11,12</sup>. Activated macrophages, are the second actors of the immune defence and replace the neutrophils in the late phase of the immune response. Activated macrophages are present in the trauma site after two-three days and can persist in

the case of infection. The multinucleated giant cells, observed sometimes at the nearest vicinity of the biomaterials, are generated from the fusion of activated macrophages and these polykaryons persist at the site of implantation for months and even years.

Neutrophils and activated macrophages can metabolize oxygen to generate the superoxide anion ( $O_2^{\bullet-}$ ) via the NADPH oxidase. This free radical is extremely reactive and can be transformed into the hydroxyl radical ( $OH^{\bullet}$ ) and initiate the oxidation of the polymer surface. Inside the cells, specific enzymes are capable of removing these free radicals; superoxide dismutase (SOD) present in the peroxisomes, can convert the  $O_2^{\bullet-}$  into hydrogen peroxide ( $H_2O_2$ ), which itself can be converted into hypochlorite acid (HOCl) by the myeloperoxidase (MPO) inside the lysosomes of the neutrophils. HOCl can oxidize the amine groups of the proteins into chloramines that is one of the sources of chloride in the body. HOCl can also oxidize the functional amino-groups (amide, urethane) with the possibility of a cleavage of these groups. The macrophages do not possess the MPO, so their  $H_2O_2$  is not converted directly into HOCl. However, neutrophils can release their active MPO into the surrounding tissue which can bind the surface of the biomaterials<sup>3,13</sup>. The  $H_2O_2$  released by the macrophages in the surrounding tissue can be catalyzed into HOCl via the MPO released by the neutrophils.

### **1.2. Degradation of ceramics**

Ceramics are extremely sensitive to variations in pH and in acidic condition ceramics such as  $\beta$ -TCP, hydroxyapatite are degradable<sup>14</sup>. It has been a major concern in the past decade to characterize the cells responsible *in vivo* for the degradation of the implanted ceramic. Degradation of a bone-implanted ceramic can be mediated by (i) inflammatory multinucleated giant cells, formed by the fusion of macrophage, which degrade the material by phagocytosis with a low-grade resorption, and (ii) osteoclasts, corresponding to physiologic multinucleated cells, involved in the resorption of bone tissue<sup>15</sup>. There has been an intense debate whether multinucleated giant cells involved in biomaterial degradation actually are osteoclast as it appeared that they do not possess all the osteoclast features<sup>16,17</sup>. To date, there has been accumulating evidence that osteoclasts are capable of resorbing calcium phosphate (CaP) ceramics *in vitro* and *in vivo*<sup>18-20</sup>. Osteoclasts cultured on CaP ceramic develop typical ultrastructural features of bone osteoclasts, such as a polarized dome shape, a clear zone and a ruffled border<sup>19,21</sup>. Modification of the shape and density of CaP crystals under the ruffled border can include an

acidic microenvironment. Moreover, osteoclasts can degrade ceramic by simultaneous resorption and phagocytic mechanisms. Many environmental factors are involved in the gradual degradation of CaP ceramic after implantation, including physicochemical processes (dissolution-precipitation) and the effect of various cell types. Heymann *et al.* (1999) reviewed the cellular mechanisms of CaP ceramic degradation<sup>22</sup>. Ceramics can be degraded by phagocytic mechanisms by cells such as fibroblasts, osteoblasts, monocytes/macrophages or by an acidic mechanism generated by a proton pump to reduce the pH of the microenvironment and resorb this synthetic substrate; the main function of an osteoclast. Osteoclasts resorb CaP ceramics in a similar process to that observed with the natural bone involving the following three phagocytic steps: (i) *Crystal phagocytosis*, (ii) *disappearance of the endophagosome envelope membrane* and (iii) *fragmentation of phagocytosed crystals in the cytoplasm*<sup>21</sup>. However, the physicochemical properties of the ceramic and mainly its solubility and composition of Ca influence this degradation process. Ceramic with a high rate of dissolution, increase the intracellular concentrations of Ca in the osteoclast which in turn results in the disorganization of the intracellular actin network present in the osteoclast podosomes and subsequent detachment of the osteoclast from the material surface.

## **2. METHODS TO ASSESS THE RESORBABILITY IN VITRO**

*In vitro* assessment of the biological resorbability is essentially determined by using cell culture techniques. The key points worth considering herein are the method to be employed to culture the cells at the surface of the material and the choice of the cell type.

### **2.1 Choice of the cell type and cell culture conditions**

One of the important parameters to take into account is the choice of a relevant cell type susceptible to be in contact with the biomaterial after implantation. For example, bone prosthesis should be tested with monocyte-macrophage, bone cells or endothelial cells which are likely to be in contact with but certainly not with brain cells.

As soon as the appropriate cell type is chosen, the next step is to choose the suitable shape and size of the material for the cell culture and analyses. The choice of the material is a key point in the *in vitro* assay. The material itself will determine the adhesion or non-adhesion of the cells at its surface. Post-implantation the surface

of the material is adsorbed by proteins circulating in the biological fluids. These proteins serve as a substrate for cell surface receptors, mainly integrins, for the cells to adhere at the surface of the material. However, the nature of the protein layer and the quantity of the protein adsorbed at the surface of the material is dependent on the surface material properties. Protein adsorption is influenced by the roughness of the surface; the rougher the surface, the greater the concentration of proteins deposited. Protein adsorption is also dependent on the chemical composition of the surface and its surface energy (influenced by the free surface energy, the zeta potential and the wettability of the surface) thus influencing the nature of the protein that could adhere on the surface. It is well known that the positively charged surfaces induce cell adhesion and proliferation whereas negatively charged surfaces exhibit a very low rate of cell adhesion. The surface charge can be modified by the pH of the medium used for the cell culture. The pH of the medium is also an important factor to take into account, as it will influence the charge of the material, and the cell behaviour. For example, mature osteoclasts are more resorbing in acidic pH than neutral or basic pH. For these reasons, it is usually necessary to pre-incubate (preferably overnightly) the material in the culture media required for the cell culture in order to allow the protein deposition on the biomaterial surface for a maximal adhesion.

The shape (e.g. disk, cylinder, beads) and the thickness of the material are also important. The choice of the shape and thickness of the material under investigation depends on the type of the study to be conducted. To visualize the cells at the surface of the material after some days in culture (albeit by light, fluorescence or scanning electron microscopy), having the material in a disk shape would be the most suitable form; facilitating the rapid fixation of the specimens on a glass slide or metal stub. Furthermore, any changes on the surface roughness (and an indicator of any degradation) can be detected on the disk by either *contact profilometry*, *scanning probe microscopy* or *image analysis with a fractal algorithm*. However, if the experimental design requires the detection of the resorption notches, the use of “round” shape materials in the form of cylinder or beads, would be preferable as it facilitates the embedding the semi-thin or ultra-thin sections.

### **3. CHARACTERIZATION OF THE RESORBABILITY IN VITRO**

The characterization of the resorbability of a material is achieved mainly by microscopic observation of the surface. It is well known that resorbability of the surface can be measured by an increase in the surface roughness. However, when the material contain specific cleavable sites, it is possible to assess the quantity of

lateral side cleaved by *biological* means, hence facilitating the quantification of the rate of resorption.

### **3.1. Microscopic analysis of the surface**

The microscopic analyses of any surface can be determined at different magnitude scales: *microscopic* levels by scanning electron microscopy, confocal and fluorescent microscopy, contact and laser profilometry with image analysis using a fractal algorithm or at the *nanoscopic* level by transmitting electron microscopy and scanning probe microscopy.

#### **3.1.1 Electron microscopies**

There are two different types of electron microscopies: scanning electron microscopy (SEM) and transmitting electron microscopy (TEM). With the SEM technique, the surface of the samples is scanned by an electron beam. The energy brought by the electron beam induces the emission of new electrons from the sample surface, mainly secondary electrons, which are collected to a specific detector for secondary electron and formed the image. However, the sample surface can emit other electron types like backscattered electron, specific of the chemical elements, auger electrons and X-electrons, the presence of specific detectors for these electrons are required to obtain the specific image. To assess whether a material is resorbable, it is recommended to remove the cells from the surface. Using SEM, it is possible to scan the sample surface before and after cell culture and compare the surface roughness. As such, if the material has been exposed to degradation by cells, the presence of *peak and valleys* on the surface induced by the enzymatic activity of the cells increases the surface roughness. However, it would be rather difficult to quantify the depth of the resorption lacunae using this approach.

With transmitting electron microscopy (TEM), the sample is embedded into a specific resins to be cut in *semi-thin* (1.5 – 2  $\mu\text{m}$  thickness) and *ultra-thin* (75-100 nm thickness) sections. Semi-thin sections are stained with usual staining like toluidine blue or Azur II to visualize the presence of the cells and eventually the presence of a notch in the material below the cell. It is worth noting that usually semi-thin sections served only as a location of the sample, and as soon as the region of interest has been identified, the sample is cut into ultra-thin sections to be observed by TEM. The ultra-thin sections is deposited on a specific metallic grid (mainly made of copper) and stained with heavy elements, mainly uranium and lead. Then the grid is introduced into the electron microscope and the electron beam will focus on the ultra-thin

section. Because of the thickness of the sample, the electrons emitted from the probe of the microscope can cross the sample and are collected on a specific detector (and only transmitted electrons). The presence of heavy elements is used to impair the electron from the source to cross the sample, so actually the image collected on the detector is a 8-bit images in grey levels, the white zone correspond of the electrons which cross the samples and the black zone to the electrons collected by the heavy elements. On a TEM image it is easy to assess the presence of notch on the surface of the biomaterial and the presence of the material inside cell vacuolations. On a TEM image, it is possible to assess the depth of the notch.

For both, SEM and TEM, the sample needs to be dehydrated and some artefacts, due to the dehydration process, can interfere with the “real” size and shape of the surface. Moreover, with the TEM technique, it is worth noting that the shape and the depth of the notch could be slightly modified by the embedding and cutting processes.

### **3.1.2. Contact microscopy**

This kind of microscopy regroups the profilometry (contact or laser profilometry) and the scanning probe microscopy [Atomic Force Microscopy – (AFM), Scanning Tunnelling Microscopy – (STM)].

#### **3.1.2.1 Profilometry**

Two different types of profilometry have been documented: the *contact profilometry* where a metallic probe is scanning the surface, and the *laser (or optical) profilometry*, where a light or laser beam is scanning the surface without direct interaction with the surface in contrast to the profilometry.

The *contact profilometry* is the oldest technique. It is a mechanical technique which uses a thin probe (2.5  $\mu\text{m}$  at its extremity) which scan the sample surface. The diameter of the probe at its extremity is the limiting factor (**Figure 5**). The moves of the probe are transmitted and amplified by a cantilever to determine the  $Z_0$  line. Below this line are defined the notch or pits at the surface of the material, above this line are defined the surface asperities.

The *optical profilometry* is a more recent and modern approach and has been developed to increase the accuracy of the contact profilometry. Briefly, a source of light is used to scan the sample surface and the light beam diffracted by the surface



roughness is collected on a mirror. The image generated is the deviation of the light beam on the mirror. With this technique, it is possible theoretically to assess the roughness as low as nanometer. However, the limitation of this technique is to possess a surface capable of reflecting the light beam and often it is necessary to modify the sample surface for a better reflection.

### 3.1.2.2 *Scanning probe microscopy*

The AFM technique is now widely used in the material science to assess the physical surface properties of a material. The principle of this technique is schematically represented in **Figure 6**. Briefly, a probe is located at the end of a microlever and scans the surface of the sample. The interactions between the probe and the sample surface induce deflections of the microlever. The deflections of the microlever are detected by an optical system: a laser beam is focused on the extremity of the microlever, just above the probe. The samples are located at the surface of a piezoelectric tube which allows displacement in the x, y and z directions. The interaction force varying from point to point (depending of the roughness of the sample), it is possible to observe deflections of the microlever. A feedback loop is used to maintain as a constant the position of the microlever compared to the sample surface and the image recorded corresponding to the current sent by the feedback loop to the piezoelectric tube to maintain constant the position of the microlever compared to the sample surface. The size of the probe is largely thinner than the one used for contact profilometry (average diameter 45-200 nm). The other advantage of the AFM technique is that it is possible to operate in a contact mode (which represents a distance of 10-100 angstroms between the end of the probe and the sample surface) or in a tapping mode, when the probe comes to the surface and is deflected from each point, with a specific vibration frequency. With this last mode it is possible also to determine differences in hardness of the surface material.

Both profilometry and AFM allow calculating the roughness coefficient (Ra), which corresponds to the average height of the peaks and valleys on the surface of the material. It is calculated using the following equation:

$$Ra = \frac{\sum_{i=1}^n |z_n|}{N}$$

Where  $|Z_n|$  is the absolute value of the difference in levels between the Z0 line and the peak or the valley for each point. N is the number of points. However, the profilometry and AFM are limited by the geometry of the sample (ex: orthopaedic

screw) and cannot be used when the geometry of the surface is complex. As such for the past 5 years, surface roughness of materials have been used intensively to characterized its degradation process<sup>23-25</sup>.

### **3.1.2.3 *Image analysis with a fractal algorithm***

Surface roughness can be evaluated by texture analysis of microscopic images using a *fractal algorithm* (the "skyscraper" algorithm)<sup>26-28</sup>. It is necessary to take pictures of the surface by episcopic illumination or by SEM. Briefly, images of the surface need to be converted and coded on 8 bits (i.e., in 256 gray levels, where black = 0 and white = 255). Pixels, which constitute an image A, can be considered as skyscrapers whose height is represented by the gray level. The roof of a skyscraper is a square of side  $\epsilon$ . The surface area of the image A( $\epsilon$ ) is obtained by measuring the sum of the top surface ( $\epsilon$ ) and sum of the exposed lateral sides of the skyscrapers.

The gray levels of adjacent pixels are then averaged in squares of  $\epsilon$ : 2, 4, 8, 16 and 32 pixels to produce new images and A( $\epsilon$ ) is calculated for each  $\epsilon$  according to:

$$A(\epsilon) = \sum \epsilon^2 + \sum \epsilon [abs[Z(x, y) - Z(x+1, y)] + abs[Z(x, y) - Z(x, y+1)]]$$

where  $Z(x,y)$  is the height of a skyscraper in the  $x, y$  plane. *Abs* is the absolute value. The fractal dimension of the surface (D) was determined by plotting a graph of  $\log A(\epsilon)$  vs.  $\log \epsilon$ . The linear regression line was computed only on the aligned points by the least-squares method. The fractal dimension was obtained as  $D = 2 - \text{slope}$ .

The more is a surface degraded the higher likelihood of the fractal dimension to reach 3. This method was successfully used to assess the surface roughness of titanium disks on SEM images and the surface roughness of hydrogel disks by episcopic illuminations<sup>26-28</sup>.

### **3.1.3 Fluorescent microscopy and biological testing**

Fluorescent microscopy employs the presence of specific molecules capable of absorbing energy of photons to re-emit the energy at a different wavelength. To increase the flexibility of the approach, increasing number of fluorescent probes have been coated on materials to assess their biodegradability. The principle of the

microscopic examination is simple; a fluorescent probe (usually conjugated to a fluorophore like FITC or Rhodamine) is deposited on the surface. When the material surface is degraded, the probe is released in the medium and the degraded surface of the material appears “non-fluorescent”. The classical example is in a study by Rogers and his team<sup>29</sup> whereby the dentine slice was exposed to a fluorophore-conjugated bisphosphonate and when the osteoclasts resorb the organic matrix the pits appeared dark whereas the rest of the dentine surface and the osteoclasts appear fluorescent.

Biological and biochemical tests can be used also to evaluate the resorption rate. These techniques are suitable to assess the degradation rate *in vivo*. The principle of the assay is to collect the supernatant of cell culture and to quantify the degradation products. For example, the resorption of dentine slice can be followed by a biochemical test, such as Enzyme Linked ImmunoSorbent Assay (ELISA), by measuring the amount of the degradation products of collagen type I. The increase in the release of the degradation products is directly proportional to the rate of the resorption process.

## FIGURES

**Figure 1:** Schematic view of the different mode of polymer and ceramic degradations.

**Figure 2:** Hydrolytic degradation of polymers.

**Figure 3:** Hydrolysable groups of polymers under certain conditions

**Figure 4:** Oxidative degradation of polymers

**Figure 5:** The size of the probe is the limiting factor of contact profilometry. Note that the probe cannot go to the end of the valley and that the surface roughness is underestimated.

**Figure 6:** Principle of the AFM microscopy. A laser can detect the deflexions of the microlever, which reflect it to the mirror. A feedback loop maintains the constant position of the microlever to the sample surface by dilating or retracting the piezoelectric tube. The movements of the piezoelectric tube are used to generate the AFM images.

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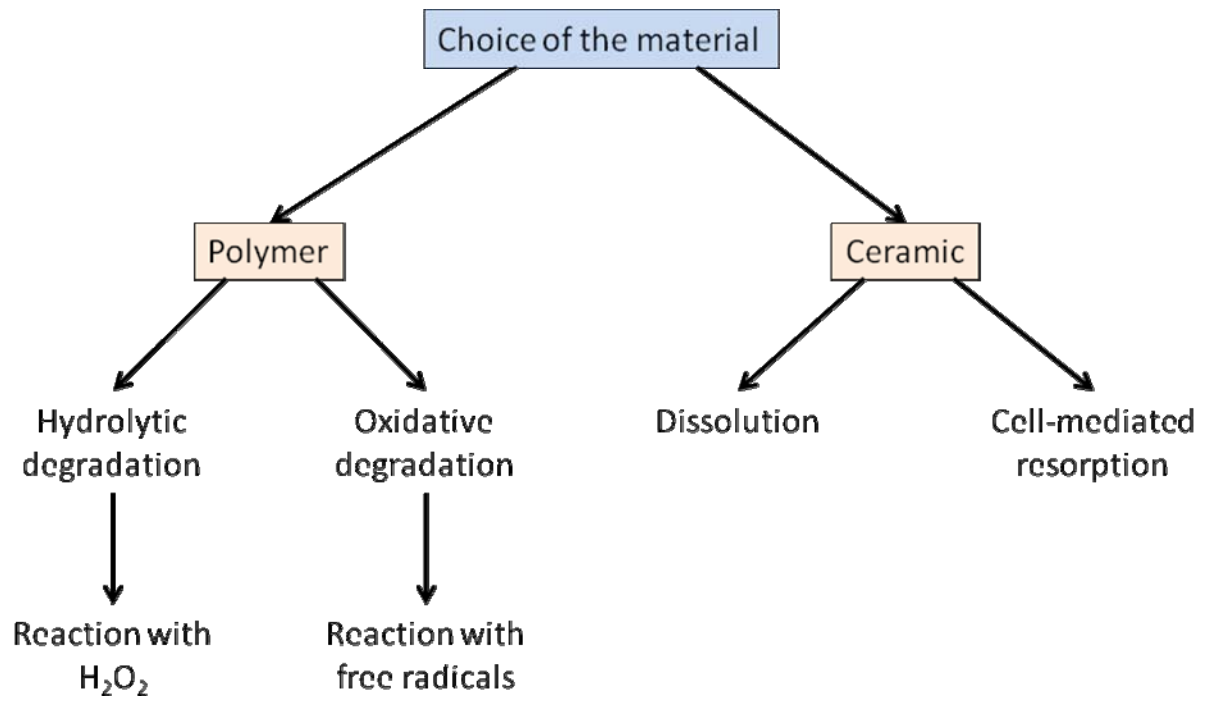
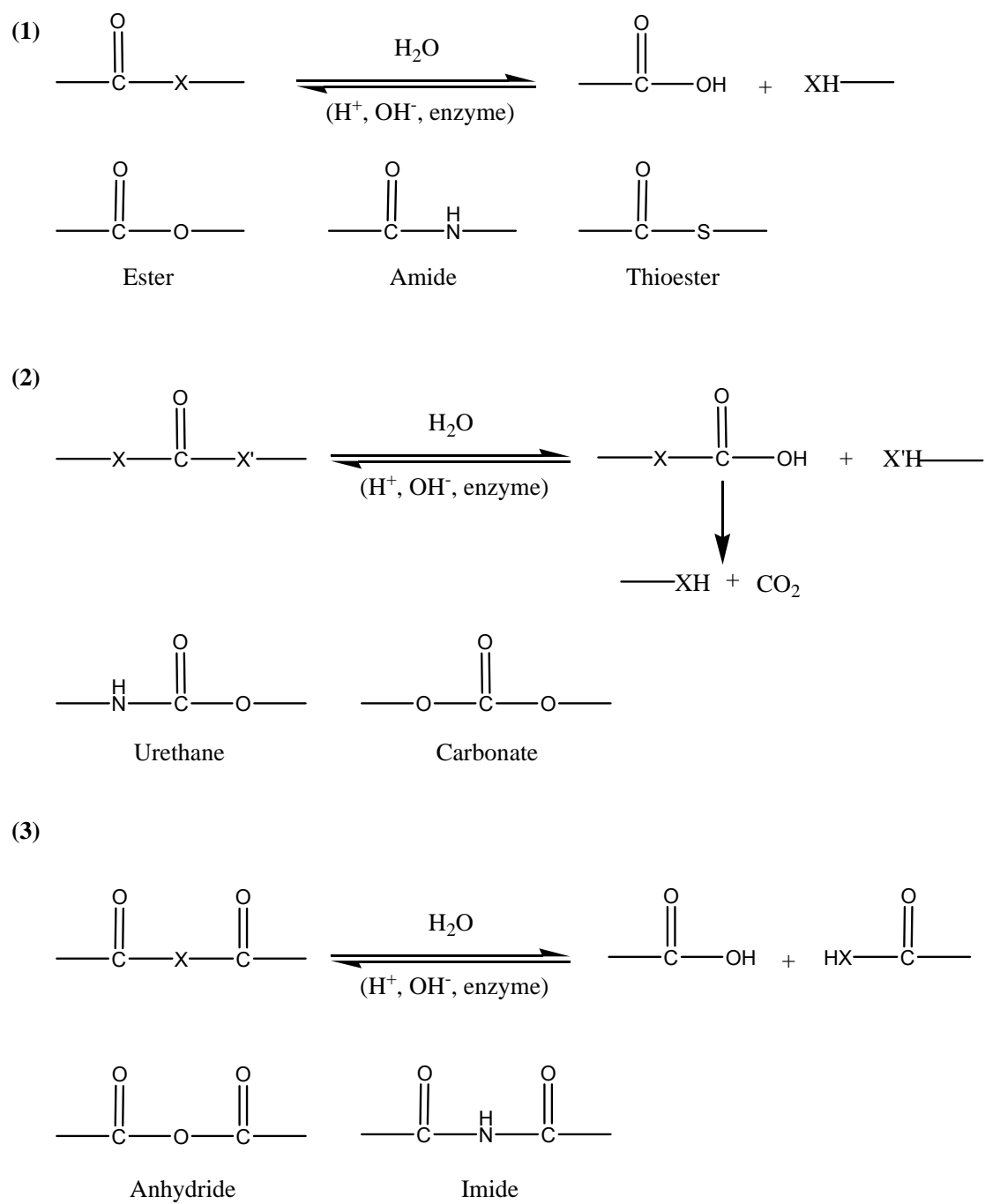
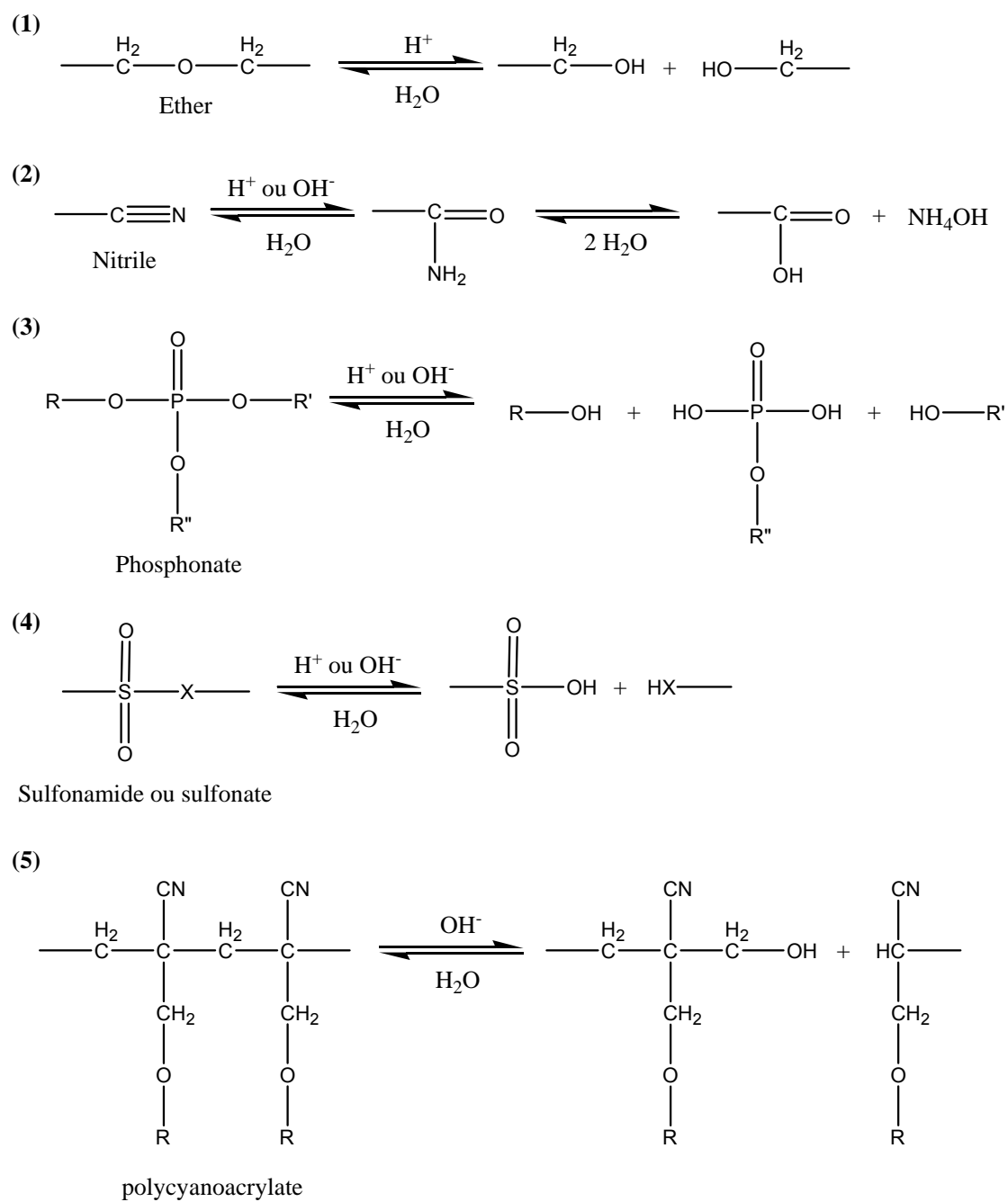


Figure 1

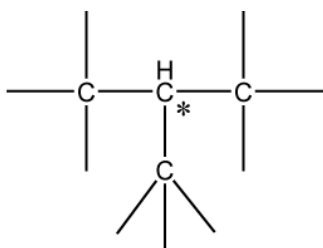




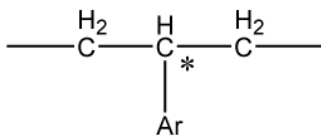
**Figure 2**



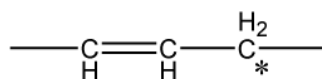
**Figure 3**



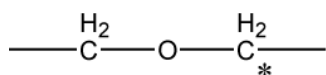
C substituted by an aliphatic chain



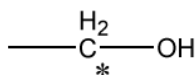
C substituted by an aromatic group



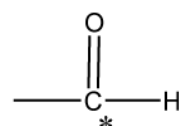
C substituted by an allylic group



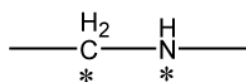
Ether



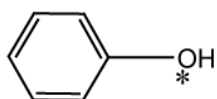
Alcohol



Aldehyde

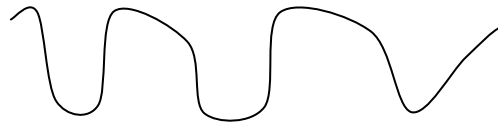
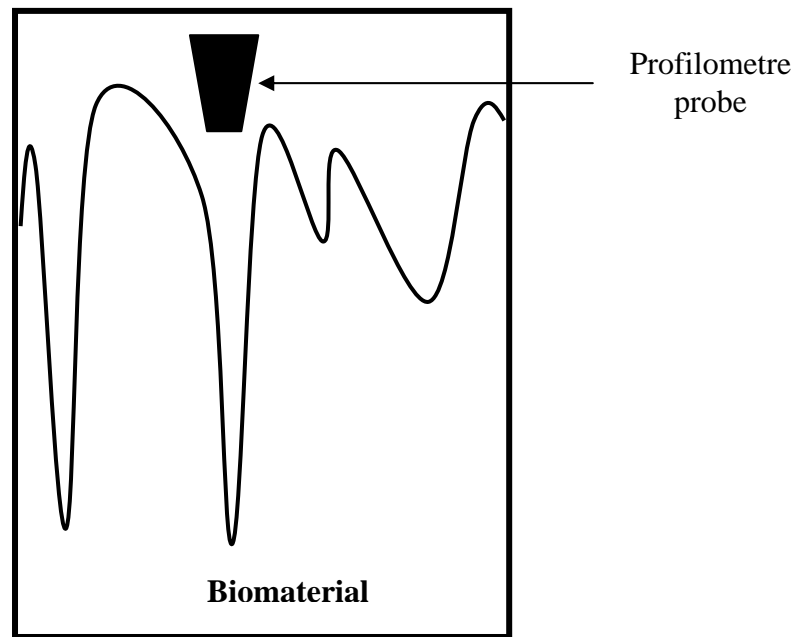


Amine



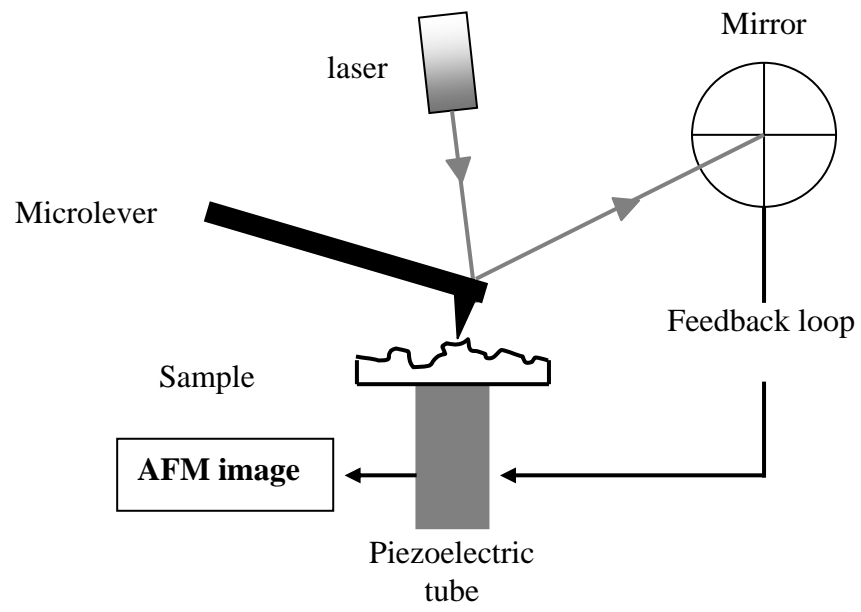
Phenol

**Figure 4**



Surface topography determined by contact profilometry

**Figure 5**



**Figure 6**

## TABLES

Anion Cation	Concentration (mM)	
	Blood	Extracellular fluid
$\text{Cl}^-$	96 – 111	112 – 120
$\text{HCO}_3^-$	16 – 31	25,3 – 29,7
$\text{HPO}_4^{2-}$	1 – 1,5	102 – 193
$\text{SO}_4^{2-}$	0,35 – 1	0,4
$\text{H}_2\text{PO}_4^{2-}$	2	
$\text{Na}^+$	131 – 155	141 – 150
$\text{Mg}^{2+}$	0,7 – 1,9	1,3
$\text{Ca}^{2+}$	1,9 – 3	1,4 – 1,55
$\text{K}^+$	35 - 56	3,5 - 4

Table1: Ionic concentration in blood and extracellular fluid <sup>30</sup>.