Evaluating the Biocompatibility of materials: Routes and strategies of exploring the host response

T. A. Tabish[1,a], M. T. Z. Butt[2,b], M.Kamran[2,c], M.Akmal[3.d], , M. Ali[4,e]

[1]Institute of Advanced Materials, Bahauddin Zakariya University, Multan 60800, Pakistan

[2]Department of Metallurgy and Materials Engineering, College of Engineering and Emerging Technologies, University of the Punjab, Lahore 5400, Pakistan

[3]Faculty of Material Science and Engineering, GIK Institute of Engineering Science and Technology, Topi 23640, Pakistan

[4]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan 60800, Pakistan

[a]engrtanvir@bzu.edu.pk

Keywords

Biomaterial, Biocompatibility, Implants, Cytotoxicity, Corrosion behavior

Abstract

A Biomaterial is a synthetic material which is used in living body as replacement of any part and its evaluation and performance is measured by its activity, stability and compatibility with living system. Usually in the field of biomaterial characterization its performance is based upon Biocompatibility. The aim of the present research is to describe the parameters and standards for evaluation of biocompatibility of biomaterials. In this study various techniques of biocompatibility testing have been described such as Cytotoxicity Testing, Genotoxicology / Mutagenicity Testing, Hemocompatibility Testing, Implantation Testing, Irritation/Intracutaneous Reactivity Testing, Pyrogenicity Testing (In Vivo), Sensitization Testing, Subacute/Subchronic Toxicity Testing, Systemic (Acute) Toxicity Testing, Chronic Toxicity and Carcinogenicity Testing, Immunogenicity testing, Pharmacokinetics and Pharmacodynamics testing, Cell Culture Test, Mucosal damage and Mucosa usage tests, Periapical tissue damage and endodontic usage test, Intraosseous Implant test, Diagnostic tests on patients, Patch test, Prick Test, Radio allegro sorbent test (RAST) and Corrosion testing. The description of the basics of these techniques along with evaluation standards is also the fundamental objective of this work.

Introduction:

A biomaterial can be defined as a synthetic material which should be pharmacologically suitable and compatible, used to replace part of a living body. The important factor which is being cared that it does not alter the properties and mechanism of living system. Either Biomaterial is used in implantation for living system or it is being used for outer surface of body it should be designed in such a way that how it influences the body system and what kind of performance it shows. Table 1 gives information regarding some examples of the uses of biomaterials, which include replacement of a body part that has lost function due to disease or trauma, to assist in healing, to improve performance, and to correct abnormalities. The role of biomaterials has been influenced considerably by advances in many areas of biotechnology and science. For example, with the advent of antibiotics, infectious disease is less of a threat than in former times, so that degenerative diseases assume a greater importance. Moreover, advances in surgical technique and instruments have permitted materials to be used in ways that were not possible previously.

Problem area	**Examples**
Replacement of diseased or damaged part	Artificial hip joint, kidney dialysis machine
Assist in healing	Sutures, bone plates, and screws
Improve function	Cardiac pacemaker, intraocular
Correct Functional abnormality	Cardiac pacemaker
Correct cosmetic problem	Augmentation mammoplasty, chin augmentation
Aid to diagnosis	Probes and catheters
Aid to treatment	Catheters, drains

Table 2. Uses Of biomaterials [2]

The success of biomaterials in the body depends on factors such as the material properties, design, and Biocompatibility of the material used, as well as other factors not under the control of the engineer, including the technique used by the surgeon, the health and condition of the patient, and the activities of the patient. Consequently, even if one failure mode such as

implant fracture is perfectly controlled so that the corresponding reliability is unity, other failure modes such as infection could severely limit the utility represented by the total reliability of the implant. One mode of failure which can occur in a biomaterial, but not in engineering materials used in other contexts, is an attack by the body's immune system on the implant [1]. Biocompatibility involves the acceptance of an artificial implant by the surrounding tissues and by the body as a whole. Biocompatible materials do not irritate the surrounding structures, do not provoke an abnormal inflammatory response, do not incite allergic or immunologic reactions, and do not cause cancer. Other compatibility characteristics which may be important in the function of an implant device made of biomaterials include (1) adequate mechanical properties such as strength, stiffness, and fatigue properties; (2) appropriate optical properties if the material is to be used in the eye, skin, or tooth; and (3) appropriate density. Sterilizability, manufacturability, long-term storage, and appropriate engineering design are also to be considered [1].

Biocompatibility refers to the ability of material to perform with an appropriate host response in a specific application. There are two main factors that determine the biocompatibility of a material: the host reactions induced by the material and the degradation of the material in the body environment [2].

Different techniques of biocompatibility

Moreover, individual test methods are usually adequate only to describe or document a single aspect of a certain type of unwanted reactions. For example, cell culture tests will detect only the influence of a material on isolated cells. These findings cannot be transferred to patients without limitation. An alloy that does not cause a reaction in cell cultures may very well result in problems in patients because there may be a lower pH value below plaque or in crevices (e.g., telescope result in a more pronounced corrosion of the alloy in vivo compared to the neutral conditions in cell cultures. However, cell culture findings may help explain the mechanisms of an unwanted reaction in a patient, for instance, an inflammation of the gingival [3]. Following are the techniques of biocompatibility testing

Cytotoxicity Testing
Genotoxicology / Mutagenicity Testing
Hemocompatibility Testing
Implantation Testing
Irritation/Intracutaneous Reactivity Testing
Pyrogenicity Testing (In Vivo)
Sensitization Testing
Subacute/Subchronic Toxicity Testing
Systemic (Acute) Toxicity Testing

Chronic Toxicity and Carcinogenicity Testing
Immunogenicity testing
Pharmacokinetics and Pharmacodynamics testing
Cell Culture Test
Mucosal damage and Mucosa usage tests
Periapical tissue damage and endodontic usage test
Intraosseous Implant test
Diagnostic tests on patients
Patch test
Prick Test
Radio allegro sorbent test (RAST)
Corrosion testing

Essential Standards and parameters for techniques of biocompatibility testing

1. Cytotoxicity Testing

The purpose of the cytotoxicity test is to determine if extracts of the test material are toxic to mammalian cells. Biocompatibility studies have shown the material should be well tolerated by cells and tissues. In a cytotoxicity evaluation no signs of harmful reactions should be detected in cell layers exposed to samples. In an in vivo biocompatibility evaluation three different methods can be used: the classical skin sensitization assay (Buehler patch test) in guinea pigs, the rabbit intracutaneous test, and the systemic injection test in mice. Samples of the material are extracted using the following solutions, with each sample being extracted with one vehicle [4].

• 0.9% Sodium Chloride (normal saline)
• Cottonseed (vegetable) oil
• Polyethylene Glycol
• 5% Alcohol in normal saline

Four rabbits are prepared by removing the hair from the injection sites and cleaning the area with alcohol. 0.2 mls of the test extract are injected intracutaneously on the right side of the exposed spinal column area at 5 sites, and 0.2 mls of control are injected on the left side of the spinal column. Each site of injection is observed immediately after injection and at twenty-four, forty-eight and seventy-two hours post injection. Prior to each observation, the skin above the injection site is saturated with dilute alcohol. The observations are scored and recorded according to the presence and degree of erythema, edema and necrosis. The average scores for erythema and edema for the test and control sites are determined at each scoring period for each rabbit. After the 72 hour scoring, an overall mean score is calculated for each test and control. If at any observation period, the average reaction to the test solution is questionably higher than the

average reaction to the control, the test is repeated using an additional three rabbits in the test group. The requirements of the test are met if the difference between the test article and control mean scores is 1.0 or less [4].

Molecular Toxicology

Molecular toxicology methods have also been introduced. For example, fluorescence-activated cell sorting (FACS) and Western blotting (a method for detecting specific proteins by gel electrophoresis, transfer to a membrane such as nitrocellulose, and detecting by antibodies) are applied to detect the influence of a biomaterial on cell metabolism, especially upon signaling pathways within the cell. This may include the determination of radical oxygen species, apoptosis rate, DNA damage and repair, changes of the cell cycle, or synthesis of specific inflammation mediators. Gene expression analysis using microarray test systems (e.g., Affymetrix) reveals information on the genes involved in the cellular stress response [5]

2. Genotoxicology / Mutagenicity Testing

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes may involve a single gene or gene segment, a block of genes or whole chromosomes. Effects on whole chromosomes may be structural and/or numerical [6].Genotoxicity is a broader term and refers to potentially harmful effects on genetic material which are not necessarily associated with mutagenicity. Thus, tests for genotoxicity include tests which provide an indication of induced damage to DNA (but not direct evidence of mutation) via effects such as unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE), DNA strand breaks, DNA adduct formation or mitotic recombination, as well as tests for mutagenicity . [7]

Therefore the SCCNFP, for the in vitro base level testing of cosmetic ingredients indicated above, recommends four assays, represented by the following test systems: [8]

For vitro evaluation the tests performed are 1. Tests for gene mutation 2. Tests for clastogenicity 3. Tests for aneugenicity and non-disjunction [9]

For In Vitro Metabolic Activation Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post mitochondrial fraction (S9) prepared from the livers of rodents (usually rat) treated with enzyme-inducing agents such as Aroclor 1254 or combination of phenobarbitone and betanaphthoflavone. The post-mitochondrial supernatant fraction is usually used at concentrations in the range from 10 to 30 percent v/v in the S9 mix. The choice and concentration of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-

mitochondrial fraction. For azo dyes and diazo compounds, using a reductive metabolic activation system may be more appropriate [10, 11]

In vivo studies, there are several reasons for mutagenicity testing beyond the in vitro base level may be required. Normally, when some concern is raised by positive results from in vitro tests, further testing may be justified. The selection of the in vivo assays cannot be defined a priori and depends on the positive results observed in the in vitro assays. Nevertheless, before undertaking any in vivo testing, a thorough review is needed of the in vitro test results of the substance (with its toxico kinetic profile), available information on its chemistry and toxicological profile, as well as data on analogous ingredients. Finally, it is obvious that a particular in vivo test should be conducted only when it can be reasonably expected from all the properties of the test substance and the proposed test protocol that the specific target tissue will be adequately exposed to the test substances and/or its metabolites. [12]

3. Hemocompatibility Testing

A review of the literature regarding models and test systems results in a lack of information concerning the test conditions and reproducibility. Therefore, where possible, tests should use an appropriate model or system, which simulates the geometry and conditions of contact of the device with blood during clinical application, including duration of contact, temperature, sterile condition and flow conditions. For devices of defined geometry the relation of surface area to test results should be evaluated. When possible, the tests should be repeated a sufficient number of times (2□/6 times, this depends on the model-specific variation coefficiency) to determine the significance of the results. First, baseline levels of parameters prior to material contact have to be described. Kinetic measurements should be preferred. Materials do exist where complement activation can be demonstrated already after 5min of exposure time. Second , conditions of exposure should be defined taking account of the intended use of the device or biomaterial. Third, a set of conditions can be suggested: A) anticoagulated (citrate and hirudin for platelets function testing and/or heparinized blood for dialysers' testing) whole blood should be collected from one normal human subject B) time of exposure: not less than 15 min, not more than 240 min) temperature: 37 8C D) flow or dynamics conditions to mimic/simulate the intended use of the device [13]. It is very important to have information about the rheological (flow) conditions, the implanted material (volume percent or squared centimeters , surface-to volume ratio).The period of exposure should not exceed 4 h because of the half-life of curdling proteins[13].

4. Implantation Testing

Implant studies are used to determine the biocompatibility of medical devices or biomaterials that directly contact living tissue other than skin (e.g. sutures, surgical ligating clips, implantable devices, etc.). [14]

In soft Tissues

For implantation test in soft tissues there is a procedure which leads to essential requirement. For implantation in subcutaneous tissue, the implants were inserted into the dorsal bilateral subcutaneous tissue of 12 adult New Zealand rabbits (body weight is 3.0kg), in a process approved by the local animal ethics committee (LAEC) at the Fourth Military Medical University, Xi'an. After the rabbits were anesthetized by intramuscularly injecting Ketamine (15mg/kg) and diazepam (0.5mg/kg), pentobarbital sodium (35mg/kg; NEMBUTAL INJECTION, Dainabot, Osaka, Japan) was injected intravenously, and the implants were inserted in the dorsal subcutaneous tissue of the rabbits to observe the response of soft tissue. During the operation, the rabbits received an intravenous infusion of saline containing Isepamic in sulphate for antibiotic.The operations were performed Under the usual sterile conditions.For implantation in musculature the implants were inserted into the dorsal muscles of 12 adult New Zealand rabbits (body weight>3.0kg) as above [15]. Euthan asia was performed on the rabbits by using carbon dioxide after 2, 6, and 12 weeks of operation (n=3 for each material and each time period), respectively. The soft tissue specimens were rinsed in sterile saline for 10min and fixed in 10% neutral buffered formalin for 48hr at a volume more than 10 times that of the block section. After dehydration and embedded in paraffin. Paraffin embedded samples were sectioned at 30m thickness with a grinding system and stained with Haematoxylin and Eosin (HE). These specimens were histopathologically observed with an optical microscope number of inflammatory cells (neutrophil, Lymphocytes and plasmacell, macrophage and polykaryocyte) and fibroblast was assessed. The identification of inflammatory cells was based on their morphology. The thickness of fibrosa was also measured [15]. With the help of the above described experimental procedure and relevant results, the implantation test can be performed.

In hard tissues

As the implantation test has been described for soft tissues, the same test for hard tissues will be different but the experimental procedure for the noted test and brief description of results are as from the research work. For hard tissue, the implants were inserted into the bilateraldistal femora of 45 adult beagles (body weight 3.5kg). This process was also approved by the local animal ethics committee (LAEC) at the Fourth Military Medical University, Xi'an. Under anesthesia with Ketamine (15mg/kg) and diazepam (0.5mg/kg), the femur was exposed through a medical incision. Two holes, separated by about 10mm apart, were carefully made in the lateral surface of the diaphysis of the femur using a low-speed dental round bur, with a physiological aline external coolant , and the implants were inserted press-fit into the metaphysic of the distal

femora. The long axis of the cylinder was perpendicular to the long axis of the femur. Two TLMcylinders after MAO and activation treatment were implanted in one leg of the beagle and one bulk Ti–6Al–4V(ELI) and another TLM cylinder were implanted in the other leg as a paired control. The wound was then sutured. Before being killed, the beagles were given acheomycin (100mg/kg/day) for 6 days. Thirty beagles were euthanized at 1, 2, 4, 8, and 12 weeks after operation (n=6 for each material and each time period), respectively. Following euthanasia, bone specimens of 1, 2, 4, 8, and 12 weeks groups were separated into bone-implant blocks, and the tissue blocks were fixed in 10% neutral buffered formalin for 2 weeks at 4◦C. After routine dehydration, embedding and slicing, the specimens were embedded in light-curing epoxy resin without decalcification. Embedded specimens were sawed perpendicular to the longitudinal axis of the cylindrical implants at a site 500m from the apical end of the implant. Specimens were ground to a thicknessof30m with a grinding system. Then the sections were stained with HE. In this way, histological sections of surrounding bone were obtained for tissue investigation with an optical microscope. The percentages of bone in contact with the implants at the interface were quantitatively evaluated by an image analyzer after tracing on a histological photograph. Thirty undecalcified specimens for each metal implant after1, 2, 4, 8, and 12 weeks were used for quantitative analysis (n=6). The other fifteen beagles were euthanized at 1, 2, 4, 8, or 12 weeks after operation (n=3 for each material and each time period), respectively.Following euthanasia ,bone specimens of 1,2,4,8,and 12 weeks groups were separated into bone-implant blocks, and the specimens were immediately immersed into pentadiol solution to fix the cell. The fixed specimens were split into two parts along the long axis of the implants. The samples were dehydrated, fixed with osmic acid again , and finally sputter coated with gold for examination under SEM [15].

Implantation tests, materials are implanted subcutaneously, intramuscularly, or in the bone of an experimental animal (rats, rabbits, etc.). After different periods of implantation of the material in the tissues (between 1 week and several months), the adjacent tissue is investigated macroscopically and microscopically. After a short implantation time (1–2 weeks), degrees of inflammation surrounding the implant will primarily be assessed. In the case of an extended implantation period, the nature and quantity of the connective encapsulation will be evaluated, too [14].

Assessment: In contrast to cell culture tests, implantation studies also provide information about the removal of toxic substances from the tissue (open system) and about the defense reaction of the entire organism, such as via an inflammatory reaction. Thus, this type of study is closer to the patients than cell culture experiments are. However, a good correlation was found between cell culture data and findings from implantation tests regarding certain dental filling materials. For instance, it provokes a pronounced tissue reaction in implantation tests, as it does in cell culture experiments. When testing alloys by means of implantation, an extended implantation period of tissue contact of more than 4 weeks is necessary [14].

5. Irritation/Intracutaneous Reactivity Testing

These tests estimate the local irritation potential of devices, materials or extracts, using sites such as skin or mucous membranes, usually in an animal model. The route of exposure (skin, eye, mucosa) and duration of contact should be analogous to the anticipated clinical use of the device, but it is often prudent to exaggerate exposure conditions somewhat to establish a margin of safety for patients. In the Intracutaneous Test, extracts of the test material and blanks are injected intradermally. The injection sites are scored for erythema and edema (redness and swelling). This procedure is recommended for devices that will have externally communicating or internal contact with the body or body fluids. It reliably detects the potential for local irritation due to chemicals that may be extracted from a biomaterial. The Primary Skin Irritation test should be considered for topical devices that have external contact with intact or breached skin. In this procedure, the test material or an extract is applied directly to intact and abraded sites on the skin of a rabbit. After a 24-hour exposure, the material is removed and the sites are scored for erythema and edema. Mucous Membrane Irritation Tests are recommended for devices that will have externally communicating contact with intact natural channels or tissues. These studies often use extracts rather than the material itself. Some common procedures include vaginal, cheek pouch and eye irritation studies [14].

6. Pyrogenicity Testing (In Vivo)

Pyrogenicity tests determine the potential of materials, extracts, and/or a finished device to induce a pyrogenic response. **USP Rabbit Pyrogen Test** is adopted for this**.** The test articles are prepared in a sterile solution, which is injected intravenously into three (3) rabbits to assess pyrogenicity. The animals are observed over a 3-hour period for an increase in body temperature. If the animals exposed to the solution do not show significant increase in body temperature, the test article passes the test. If any single animal of the three has a temperature increase above the acceptable range, the test can be continued with 5 additional animals. And the sample requirement for this test is: Transfusion / Infusion Assemblies and Similar Devices: 10 device assemblies to represent the lot under test. Blood and Tissue Contact Devices: 10 devices to represent the lot under test and its turnaround time will be 22 days [16].

7. Sensitization Testing

Sensitization studies help to determine whether a material contains chemicals that cause adverse local or systemic effects after repeated or prolonged exposure. These allergic or hypersensitivity reactions involve immunologic mechanisms. Studies to determine sensitization

potential may be performed using either specific chemicals from the test material, the test material itself, or most often, extracts of the test material. The Materials Biocompatibility Matrix recommends sensitization testing for all classes of medical devices. The Guinea Pig Maximization Test (Magnusson Kligman Method) is recommended for devices that will have externally communicating or internal contact with the body or body fluids. In this study the test material is mixed with complete Freund's adjuvant (CFA) to enhance the skin sensitization response. The Closed Patch Test involves multiple topical doses and is recommended for devices that will contact unbroken skin only. The Murine Local Lymph Node Assay (LLNA) determines the quantitative increase in lymphocytes in response to a sensitizer. If a molecule acts as a skin sensitizer, it will induce the epidermal Langherhans cells to transport the allergen to the draining lymph nodes, which in turn causes T-lymphocytes to proliferate and differentiate. This method may only be used for chemicals that come into direct contact with intact skin or are transported through the skin. Additionally, this method can only reliably detect moderate to strong sensitizers. From an animal welfare perspective, this test is preferable to the Guinea Pig Maximization or the Closed Patch Test, and it allows for faster turnaround time. However, if a negative result is seen in the LLNA test, a Guinea Pig Maximization test must be conducted [14].

8. Subacute/Subchronic Toxicity Testing

Tests for subchronic toxicity are used to determine potentially harmful effects from longer-term or multiple exposures to test materials and/or extracts during a period of up to 10% of the total lifespan of the test animal (e.g. up to 90 days in rats). Actual use conditions of a medical device need to be taken into account when selecting an animal model for subchronic toxicity. Appropriate animal models are determined on a case-by-case basis. Pacific Bio Labs gives two protocols for subchronic testing that are appropriate for many devices. They may use intraperitoneal administration of an extract of the device or device material or an intravenous route of administration. Implant tests are often performed for different durations appropriate to assess subchronic toxicity of devices and device materials. Subchronic tests are required for all permanent devices and should be considered for those with prolonged contact with internal tissues [14].

9. Systemic (Acute) Toxicity Testing

By using extracts of the device or device material, the Acute Systemic Toxicity test detects leachables that produce systemic (as opposed to local) toxic effects. The extracts of the test material and negative control blanks are injected into mice (intravenously or intraperitoneally,

depending on the extracting media). The mice are observed for toxic signs just after injection and at four other time points. The Materials Biocompatibility Matrix recommends this test for all blood contact devices. It may also be appropriate for any other device that contacts internal tissues. The Material Mediated Pyrogen test evaluates the potential of a material to cause a pyrogenic response, or fever, when introduced into the blood. Lot release testing for pyrogenicity is done in vitro using the bacterial endotoxin (LAL) test. It must be validated for each device or material. However, for assessing biocompatibility, the rabbit pyrogen test is preferred. The rabbit test, in addition to detecting bacterial endotoxins, is sensitive to material-mediated pyrogens that may be found in test materials or extracts [14].

10. Chronic Toxicity and Carcinogenicity Testing

These tests are often long-term studies that can extend for a period of up to two years or longer. If the device involves new chemistry that (from material characterization and exposure assessments) indicate a high enough risk, one or more of these studies may be necessary. If this is the case, contact your Account Manager for assistance in designing an appropriate long-term study [16].

11. Immunogenicity testing

The immunogenic potential of a biotherapeutic is defined as its ability to provoke an immune response, either by humoral production of anti-drug antibodies (ADA) or through cellularbased immune responses [17, 18, 19]. Testing strategy for this test is that the Immunogenicity testing should be done on the basis of a risk assessment and is usually done in several stages and the Screening assay that detects all antibodies binding to the biopharmaceutical (ADA; anti-drug antibodies) in serum samples of animals or patients, confirmatory assay should be performed to eliminate false positive samples, ASSAY For neutralizing antibodies that detects those serum samples that contain neutralizing antibodies, and characterization of anti-drug antibodies detected in serum samples. Screening for ADA is usually done using suitable ELISA formats, radio-immune precipitation (RIPA), e.g. with 125I labeled antibodies, or surface plasmon resonance (SPR). The same assays are used for the confirmation step, e.g. by demonstrating inhibition of binding by excess of the drug. Bridging ELISA formats are particularly useful in early project stages and for different animal species when project specific immunological reagents are not available. To run a bridging ELISA, labeled drug is needed, however. The label may be detected directly (e.g. fluoresceine) or indirectly via an amplification system [20].

12. Pharmacokinetics testing and Pharmacodynamics testing

Pharmacokinetic studies based on a traditional intensive design model are usually conducted using carefully selected volunteer subjects, a controlled experimental design, and collection of multiple blood samples.After measurement of drug and metabolite concentrations in all samples, pharamacokinetic models are applied to determine parameters such as elimination half-life, volume of distribution, and clearance [21].

In vivo studies

For certain medicines and dosage forms, in vivo documentation of equivalence, through either a pharmacokinetic bioequivalence study, a comparative pharmacodynamic study, or a comparative clinical trial, is regarded as especially important. In vivo documentation of equivalence is needed when there is a risk that possible differences in bioavailability may result in therapeutic inequivalence [22]. Examples of this one are given as. (a) Oral immediate release pharmaceutical products with systemic action when one or more of the following criteria apply: critical use medicines; narrow therapeutic range (efficacy/safety margins); steep dose-response curve; documented evidence for bioavailability problems or bio-inequivalence related to the active pharmaceutical ingredient or APIs of similar chemical structure or formulations (unrelated to dissolution problems); there is scientific evidence to suggest that either the polymorphs of API, the excipients or the pharmaceutical processes used in manufacturing could affect the bioequivalence. (b) Non-oral and non-parenteral pharmaceutical products designed to act by systemic absorption (such as transdermal patches, suppositories, nicotine chewing gum, testosterone gel and skin-inserted contraceptives, etc.). (c) Modified release pharmaceutical products designed to act by systemic absorption. (d) Fixed combination products with systemic action, where at least one of the active pharmaceutical ingredients requires an in vivo study. [23] (e) Non-solution pharmaceutical products, which are for non-systemic use (oral, nasal, ocular, dermal, rectal, vaginal, etc., application) and are intended to act without systemic absorption. In these cases, the equivalence is established through, e.g. comparative clinical or pharmacodynamic, dermatopharmacokinetic studies and/or in vitro studies. In certain cases, active pharmaceutical ingredient concentration measurement can be still required for safety reasons in order to assess unintended systemic absorption [24].

In vitro studies

The Biopharmaceutics Classification System (BCS) is based on aqueous solubility and intestinal permeability of the drug substance. It classifies the active pharmaceutical ingredient (API) into one of four classes: Class 1- High Solubility, High Permeability Class 2 - Low Solubility, High Permeability Class 3 - High Solubility, Low Permeability Class 4 - Low Solubility, Low Permeability. By combining the dissolution of the drug product with these two properties of the API, the three major factors that govern the rate and extent of drug absorption

from immediate release solid dosage forms are taken into account. With respect to dissolution properties, immediate release dosage forms can be categorized as having “very rapid”, “rapid”, or “not rapid” dissolution characteristics. On the basis of scientific principles of solubility and permeability and dissolution characteristics of the dosage form, the BCS approach provides an opportunity to waive in vivo pharmacokinetic bioequivalence testing for certain categories of immediate release drug products. Oral drug products not eligible for a so-called “biowaiver” based on the BCS approach. Acceptable alternative test methods for permeability determination of the drug substance include: (i) in vitro permeation using excised human or animal intestinal tissue. When one of these two alternative methods is used for permeation studies, suitability of the methodology should be demonstrated, including determination of permeability relative to the permeability of a reference compound whose fraction dose absorbed has been documented to be at least 85%. Supportive data can be provided by some additional test methods like as (ii) in vitro permeation across a monolayer of cultured epithelial cells (e.g. Caco-2) using a method validated using APIs with known permeabilities [25,26,24].

13. Cell Culture Test

The biological properties of the samples were evaluated by preliminary in vitro cell tests. The MC3T3-E1 cells were used to characterize the proliferation and differentiation behavior of the cells .Osteoblasts are cultured upon the test substrates .The culture mediumwas45%□-MEM(Gibco),45%MEM(Gibco),5%calfserum (Eurobio), 5% fetal bovine serum (Eurobio). The medium also contained gentamicin (50g/ml) and amphotericin -B(250□g/ml).Cell Proliferation is performed in 24 – muti well plates, and1 ×105cm−2 growing cells contained in 1 m l culture medium are seeded in each well on the test samples and incubated at 37◦C and 5% CO2 atmosphere with 95% humidity. 3h enables complete adhesion of the cells on the test samples, the wells are carefully filled to 1ml. The culture plate was transferred gently to a 37◦C incubator. The incubation time durations were 2, 4 and 7 days. After each time point, the samples were taken out and rinsed with a phosphate-buffered saline solution (pH 7.2, PBS) twice to remove unattached cells. Cells grown on the test samples are fixed with sodium phosphate buffered with 2.5% glutaraldehyde. After two washes in the same buffer, the cells are post fixed with1% OSO4 in saturated HgCl2.After dehydration in graded ethanol, the cells were dried to the critical point, coated with gold and examined in a scanning electron microscope [27].

14. Mucosal damage and Mucosa usage tests

Various cell cultures and animal models have been described in the literature for testing mucosal compatibility (oral mucosa test). A relatively new model consists of in vitro grown skin equivalents and is already being applied for test purposes in the cosmetics industry [28]. For

example as in vitro co-cultures are grown that consists of skin fibroblasts and keratinocytes [28]. Partially or completely differentiated, multilayered, epithelial-like cells are being used in other models [29] are being developed. And its assessment is essential because of their technical limitations, oral mucosa tests are not considered in most national and international standards, so the number of relevant publications is comparatively small. Alternatively, other test methods (cell cultures, implantation tests) can be used to determine potential damage of the mucosa. Based on the experience of the cosmetics industry, in vitro grown mucosa equivalents may offer an interesting perspective, but experiences with dental materials are still minor [30].

15. Periapical tissue damage and endodontic usage test

The literature includes descriptions of animal models (e.g., primates, dogs) that allow the application of a given material into the root canal according to endodontic techniques after a usual root canal preparation [30]. Compatibility is assessed by histologic evaluation of the periapical tissues. It is also possible to induce pulp gangrene as a disease model in the experimental animal and to perform an appropriate treatment [31].

Assessment: The classic endodontic usage test is very elaborate and includes the same technical and ethical problems as the pulp/dentin test using large experimental animals. Relatively few studies using this test method are available in the literature. The presented findings, however, document a good correlation with clinical observations. In particular, stimulating effects on special cells can be determined, such as the influence of calcium hydroxide compounds on periapical cementoblasts [32]. Otherwise, implantation tests, in which Teflon tubes are filled with the experimental material and subsequently implanted, may be used as alternatives [30].

16. Intraosseous Implant test

Materials used for dental implants are inserted into the jaw of test animals (intraosseous implants). For this, penetration of the epithelial barrier, equivalent to the treatment of patients, is simulated on experimental animals. Appropriate animals are, among others, primates, dogs, miniature pigs, guinea pigs, and rats. Tissue reaction is assessed histologically, with the tissue in contact with the implant being of particular interest [30].

Assessment: Available data from these animal studies show that implants based on titanium or ceramics, for example, are generally well tolerated by the surrounding tissue. A good correlation of these findings with patients' situations can be expressed [30].

17. Diagnostic tests on patients

Contrary to the test methods that are used to characterize a (new) material and which have been described so far, diagnostic tests on patients are used to more deeply analyze claimed or real unwanted side effects in individual subjects (individual compatibility). This branch of biocompatibility studies has become very important during recent years, since many materials do not cause clinically manifest reactions in the vast majority of the population but may generate claimed or real disease symptoms linked to materials in single cases. The assumption of an individual compatibility for dental materials is based on these observations. Thus, examination of the individual compatibility of various materials has been attempted by means of one or more test methods in order to find a feasible explanation for certain symptoms, to perform a causal treatment, or, if possible, to avoid such symptoms by a preceding examination. A variety of methods have been described, some of which are accepted by the scientific community and some that have not yet been scientifically approved. The most important methods will be discussed and critically evaluated in the following sections. Other methods, such as the analysis of blood or urine to determine exposure to heavy metals, are part of general toxicology or occupational medicine. The metal concentration of whole blood, blood plasma, or 24-h urine can be analyzed by common chemical procedures (e.g., atomic absorption spectrometry). These methods are explained in detail in textbooks of toxicology or occupational medicine [30].

18. Patch test

The patch test, originally developed and described by Jadassohn is the most important allergy test regarding dental materials. This test can be applied to identify delayed type hypersensitivity (type IV reactions) as the cause for an allergic contact dermatitis. Immediate reactions (type I reaction, such as asthma) can be diagnosed by the prick test [30]. Adhesive tapes containing the potential allergens at concentrations that are just high enough to trigger the allergic reaction (but which are nonirritating) are adhered to the clinically sound skin of the patient's back. The most important allergens are combined in so-called standard series at ready-made concentrations and are commercially available. Special series include dental materials. The patient should avoid excessive sweating or exposure to sun as well as scratching of the back, and should not have a shower or bath. During the following days, after the tape has been removed, the skin is evaluated for test reactions: redness, itching, blisters, etc. Skin reactions are assessed after 2 and 3 days but later checks (after 5 and 7 days) are also necessary to detect late reactions, since immunocompetent T lymphocytes occasionally require several days before they cause a visible allergic reaction [30].

Assessment: The patch test is the primary method for the detection of a delayed-type hypersensitivity (type IV reaction) allergy to dental materials. Although many attempts have been made, cell cultures are not yet generally accepted for diagnosing a type IV hypersensitivity (see below). Skin and oral mucosa react similarly in the case of an allergy, as in many other diseases, too. The skin is, therefore, an adequate organ for the appropriate allergy tests. The basic requirement for the stimulating effect on T lymphocytes is that the allergen is released from the material in sufficiently high quantities and then penetrates the skin [30].

19. Prick Test

This test is used to detect "immediate-type" allergies (type I reactions). The allergen is applied as a drop to the skin, and then the skin is "pricked" through the drop [30]. Test substances/extracts (e.g containing natural latex offered by various manufactures. After 5–30 min, the skin reaction is assessed (redness, formation of weals, etc. Although the risk of provoking an immediate allergic reaction by the test itself is very minor, it cannot be completely excluded. Therefore, this test should be executed only by qualified personnel. The risk of sensitization of a patient by the prick test is considered low [30].

20. Radio allegro sorbent test (RAST)

The RAST belongs to the group of in vitro tests for diagnosing an allergy. It is used to diagnose immediate-type allergies (IgE mediated) by identifying an allergen-specific IgE in the patient's blood [34]. Because the RAST is an in vitro test, the patient will not be exposed to the risk of sensitization by the test itself. However, with atopic patients or through other circulating antibodies, this test may render results that are inconsistent with clinical findings. However, with atopic patients or through other circulating antibodies, this test may render results that are inconsistent with clinical findings [35].

21. Corrosion testing

The corrosion resistance of metal alloys is based on a passivation phenomenon, which arises as a result of the metal oxide layer that forms on the surface of the metal. The corrosion trends of a material regarding biocompatibility can be checked by choosing biomedical solution such as 0.9 % NaCl solution, in which corrosion response of host is evaluated. The corrosion behavior of biomaterials can be performed by Tafel scan, Electrochemical Imepence Spectroscopy (EIS), Cyclic polarization and Electrostatic Resistance (ESR) and for these techniques Gamry software is used.

Outlook

The aims of the present research were to describe the parameters and standards for evaluation of biocompatibility of biomaterials and to compare the effect of implantation on the strength properties of material suture. In this study various techniques of biocompatibility testing have been described such as Cytotoxicity Testing, Genotoxicology / Mutagenicity Testing, Hemocompatibility Testing, Implantation Testing, Irritation/Intracutaneous Reactivity Testing, Pyrogenicity Testing (In Vivo), Sensitization Testing, Subacute/Subchronic Toxicity Testing, Systemic (Acute) Toxicity Testing, Chronic Toxicity and Carcinogenicity Testing, Immunogenicity testing, Pharmacokinetics and Pharmacodynamics testing, Cell Culture Test, Mucosal damage and Mucosa usage tests, Periapical tissue damage and endodontic usage test, Intraosseous Implant test, Diagnostic tests on patients, Patch test, Prick Test, Radio allegro sorbent test (RAST) and Corrosion testing and also basic concepts resulting from research are given. To support the standards of techniques experimental data from relevant research papers, medical devices manufacturer brushers and bio lab where these tests are professionally performed are given.

References

1. Joon B. Park and Joseph D. Bronzino (editors), Biomaterials : principles and applications, CRC press USA, 4 (2002)
2. Sauli Kujala, Biocompatibility and biomechanical aspects of nitinol shape memory metal implants, University of Oulu. 29 (2003)
3. G. Schmalz , D. Arenholt-Bindslev, Biocompatibility of dental Materials, Springer-Verlag Berlin Heidelberg. 13 (2009)
4. XDA Biocompatibility Validation Summary, Xcellerex, Inc. Marlborough, MA 01752, (2004)
5. G. Schmalz , D. Arenholt-Bindslev, Biocompatibility of dental Materials, Springer-Verlag Berlin Heidelberg. Page no (2009)
6. Proposal for Recommended Mutagenicity / Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC
7. EUROPEAN CHEMICAL BUREAU: Technical Guidance Document on Risk Assessment. European Commission, EUR 20418EN, April (2003)
8. Proposal for Recommended Mutagenicity / Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC, (1998)
9. PARRY, J.M.A proposal for a new OECD Guideline for the in vitro micronucleus test. EEMS.(1997)

10. MATSUSHIMA,T, Factors modulating mutagenicity microbial tests.(Ed.). Norpoth, K.H. and Garner, R.C. Short-term test systems for detecting carcinogens, New York, 273-285, (1980)
11. PRIVAL, M.J, Mutagenicity of Benzidine and Benzidine-congener dyes and selected monoazo dyes in a modified Salmonella assay. Mutation Research 136:33-47, (1984)
12. SCCNFP "Proposal for Recommended Mutagenicity / Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC, (1998)
13. Theo Seyfert , Volker Biehl , Joachim Schenk, In vitro hemocompatibility testing of biomaterials according to the ISO 10993-4 Ulrich, Biomolecular Engineering 19, 94 (2002)
14. Assessing Biocompatibility (Booklet), A guide for medical devices manufacturers, Pacific bio labs, San Francisco Bay Area, (2001)
15. Sen Yua, Zhentao Yua, Gui Wang, Jianye Hana, Xiqun Maa, Matthew S. Dargusch, Biocompatibility and osteoconduction of active porous calcium–phosphate films on a novel Ti–3Zr–2Sn–3Mo–25Nb biomedical alloy, Colloids and Surfaces B: Biointerfaces 85, 105 (2011)
16. WuXi AppTec, Approved documents, Biocompatibility testing, website http://www.wuxiapptec.com/news_medical-device_industry_oct-2012.html visited on 19-04-2013
17. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clin Therapeutics 24(11), 1720-40, (2002)
18. Koren E, Immune responses to therapeutic proteins in humans – clinical significance, assessments, and prediction. Curr Pharm Biotechnol 3:349-60 (2002)
19. Schellekens H, The immunogenicity of biopharmaceuticals. Neurology 61: S11-12. (2003)
20. EMEA CHMP/BMWP/14327/2006; Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins April, (2008)
21. David J. Greenblatt, Lisa L.Vonmoltke, Jerold S.Harmatz and Richard I.Shader, Pharmacokinetics, pharmacodynamics and drug disposition, K.L. Davis, Dennis Charney, Joseph T. Coyle, Charles B. Nemeroff (Editors) Neuropsychopharmacology: the fifth generation of progress An Official Publication of the American College of Neuro psychopharmacology**,**507 (2002)
22. HHS/FDA Guidance for Industry: Bioavailability and Bioequivalence Studies for Orally Administered Drug Products - General Considerations (2003)
23. WHO, FDC guide, TRS 929, Annex 5, (2005)
24. Multisource (generic) pharmaceutical products: guidelines on registration requirements to establish interchangeability, world health organization working document QAS/04.093/rev.4, (2003)
25. HHS/FDA Guidance for Industry: Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system, (2000)

26. Yu LX, Amidon GL, Polli JE, et al. Biopharmaceutics Classification System: The scientific basis for biowaiver extensions. Pharm, 19: 921-925, (2002)
27. Sen Yua,b, Zhentao Yua, Gui Wangb, Jianye Hana, Xiqun Maa and Matthew S. Darguschb, Biocompatibility and osteoconduction of active porous calcium–phosphate films on a novel Ti–3Zr–2Sn–3Mo–25Nb biomedical alloy, Colloids and Surfaces B: Biointerfaces 85, 105, (2011)
28. Schmalz, G., Arenholt-Bindslev, D., Hiller, K.-A., Schweikl, H. Epithelium-fibroblast co-culture for assessing the mucosal irritancy of metals used in dentistry. Eur J Oral Sci 105, 86–91 (1997)
29. Schmalz, G., Schweikl, H., Hiller, K.-A.: Release of prostaglandin E2, IL-6 and IL-8 from human oral epithelial culture models after exposure to compounds of dental materials. Eur J Oral Sci 108, 442–448 (2000)
30. G. Schmalz , D. Arenholt-Bindslev, Biocompatibility of dental Materials, Springer-Verlag Berlin Heidelberg. 23 (2009)
31. Fouad, A.F., Walton, R.E., Rittman, B.R.: Healing of induced periapical lesions in ferret canines. J Endod 19, 123–129 (1993)
32. Tagger, M., Tagger, E.: Periapical reactions to calcium hydroxide containing sealers and AH26 in monkeys. Endod Dent Traumatol5, 139–146 (1989)
33. Korting, H.C., Sterry, W. Diagnostic Methods in Dermatology, Blackwell Wissenschafts-Verlag, Berlin (1997)
34. Korting, H.C., Sterry, W, Diagnostic Methods in Dermatology, Blackwell Wissenschafts-Verlag, Berlin (1997)
35. G. Schmalz , D. Arenholt-Bindslev, Biocompatibility of dental Materials, Springer-Verlag Berlin Heidelberg. 33 (2009)