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Biocompatibility of polymer-based biomaterials and medical devices – regulations, *in vitro* screening and risk-management

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Biomaterials play an increasing role in modern health care systems. Biocompatibility poses a significant challenge for manufacturers of medical devices and contemporary intelligent drug delivery technologies from materials development to market approval. Despite a highly regulated environment, biocompatibility evaluation of biomaterials for medical devices is a complex task related to various factors that include mainly chemical nature and physical properties of the material, the contact tissue and duration of contact. Although international standards, such as ISO 10993-1, are generally employed to prove regulatory compliance needed for market clearance or for initiating clinical investigations, they may not offer sufficient guidance, or risk-management perspective when it comes to choosing materials or appropriate *in vitro* biocompatibility screening methods when developing medical devices. The global normative approach towards the biocompatibility evaluation of medical devices is presented in this review, with a focus on *in vitro* studies. Indeed, a risk-management approach towards the judicial choice of *in vitro* tests throughout the development and production of medical devices and drug delivery systems will facilitate rapid regulatory approval, avoid unnecessary animal studies, and ultimately reduce risks for patients. A detailed overview towards the construction of a comprehensive biological evaluation plan is described herein, with a focus on polymer-based materials used in medical applications. Polymeric materials offer a broad spectrum of applications in the manufacturing of medical devices. They are extensively employed within both conventional and innovative drug delivery systems with superior attributes supporting robust, extended use capacity, capable of meeting specific requirement such as adhesion, drug release, and more. Various methods of biocompatibility assessment are detailed within, with an emphasis on scientific analysis. This review may be of interest to those involved in the design, manufacturing and *in vitro* testing of medical devices and innovative drug delivery technologies, specifically with respect to a risk-management approach towards the biocompatibility assessment of polymer-based devices.

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1. Introduction

Medical devices, combined products and innovative drug delivery systems composed of biocompatible materials are used to interact with the human body to evaluate, replace, treat, or modify a specified function, tissue or organ. Metallic, polymeric, ceramic or composite biomaterials can be natural-based products or completely synthetic. Their biological compatibility should be assessed before a final device reaches the market and used clinically.[1–5] The choice of materials and biocompatibility studies leading towards the manufacturing and ultimately market approval of medical devices is a challenging enterprise today. International standards, such as ISO 10993-1, are generally employed to prove regulatory compliance needed for market clearance or for initiating clinical investigations. However, such standards may not represent sufficient guidance, nor a risk-management perspective when it comes to choosing materials or appropriate *in vitro* biocompatibility screening methods when developing medical devices. A risk-management approach towards the judicial choice of *in vitro* tests throughout the development and production of medical devices will facilitate rapid regulatory approval, avoid

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unnecessary animal studies, and ultimately reduce risks for patients.

The majority of materials used in medical devices are composed of synthetic polymers such as polyurethane, polyethylene, polypropylene, polystyrene, polyester, polycarbonate, polyvinyl chloride, polyacrylate, elastomers, fluoropolymers, silicone or polyethylene terephthalate. In the case of drug delivery systems, polymers represent one of the major classes of materials employed; examples include poly-acrylates, polyesters, isopropyl-acrylamides, poly(2-oxazoline)s, polyethylenimines as well as naturally-based polymers (collagen and chitosan) and poly(ethylene glycol)s for hydrogels formulation.

Polymer properties are dictated by the chemical structure, intra- and intermolecular forces that govern molecular organization, surface state and morphological characteristics. Compared with materials such as ceramics and metals, polymers are prepared in versatile compositions/structure allowing high control of their properties. With easy manufacturability at reasonable cost, polymers can be produced in various compositions, formats and quantities to suit specific medical applications with the desired mechanical and physical properties.[3,4] Such formats include particles, capsules, fibers, films, membranes, sheets, and 3D-structures (scaffolds).

Based on their behaviour in contact with living tissues, polymeric biomaterials can be divided into biostable, bioadsorbable (degradable or resorbable) and partially bioadsorbable categories. In contrast with biostable polymers, which are inert and retain their properties for years, bioadsorbable polymers have only temporarily action and gradually decompose, as with certain surgical fixation materials.[6]

Medical applications of polymers include personal protection equipment, surgical instruments, implantable devices, biological liquids transfer and storage systems, artificial organs, sutures, drug-delivery systems, carriers of cells or enzymes, microfluidic devices, biosensors, *in vitro* diagnosis tools, bio-adhesives; orthopaedic devices and many others.[7] It is worth noting that polymers used for *in vitro* diagnostic devices or products, although classified as medical devices, do not require the same process of market approval and risk analysis for biocompatibility assessment.

The main challenge of using synthetic polymers for biomedical applications is the general lack of biocompatibility, often associated with inflammatory reactions and fibrous encapsulation.[3]

In direct relation with surface contact aspects, the concept of biocompatibility is essential in the field of biomaterials. Formerly defined as material property to be neutral (physically, chemically and physiologically), biocompatibility was officially redefined in the conference of the European Society of Biomaterials (1986) as "the ability of a material to perform with an appropriate host response in a specific application".[1,2,8] This expanded definition of biocompatibility includes the notion of "bioactivity" in which the material induces a desired action in the living tissue, and thus would not necessarily be inert. This is the case, for example, with absorbable sutures where an inflammatory reaction is involved in the resorption process, as well as the case of osteoconductive devices where the material is required to interact favourably with the tissue in order to facilitate bone growth.[9] Functional materials like polymers that degrade or respond to environmental conditions, can allow a drug release by external physical stimuli (stress, electricity, light irradiation, tempera-

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ture changes) or by a change in physiological status *via* internal chemical or biochemical triggers (pH, metabolites, antigens or enzyme presences).[1–4]

Biocompatibility evaluations aim to predict whether a material could present any potential danger for patients through the assessment of conditions closely similar to the clinical situation. The diversity of biomaterials, biological applications and the nature of interaction, varying from short skin contact to long term implantation, render harmonized assessment very difficult.[10]

The host tissue response to an implanted device, prosthesis or biomaterial is a complex phenomenon ending normally with wound healing. Host response to biomaterial contact starts with protein adsorption to the surface followed by inflammatory and/or immune reactions, formation of granulation tissue leading to the healing process characterized by an equilibrium between the biomaterial and its biological environment and the formation of a fibrous envelope.[1,11]

Understanding material properties and the associated body tissue response will guide the choice of specific biocompatibility tests. This biological evaluation of medical devices is in fact a risk assessment – based strategy founded on an experimental approach.

A major focus of this review is the biological safety requirements for medical devices and the current state-of-the-art for assuring those requirements. This review outlines best practices of biological evaluation in detail. It emphasizes the risk management aspects of biocompatibility and *in vitro* testing of biomaterials and medical devices, and highlights the need for a rational approach to ultimately ensure safe use. Based on our experience in the field, the review emphasizes the evaluation of polymer-based biomaterials employed as components of medical devices and drug delivery systems.

2. Standards and regulations for biological evaluation of medical devices

The biocompatibility evaluation of medical devices and implantable drug delivery systems is a multi-stage approach, beginning with the initial screening of new materials, non-clinical and clinical pre-market safety evaluations, to product release testing, and product periodic audits. This sequence allows the concerned products to meet current international standards.

Biological evaluation of medical devices is regulated by internationally recognised standards including International Organization for Standardization (ISO) standard ISO-10993, recent FDA guidance "Use of International Standard ISO-10993", "Biological Evaluation of Medical Devices Part 1: Evaluation and Testing", and Japanese Ministry of Health, Labor and Welfare Notifications and Ordinances.

The ISO-10993 guidelines are intended to help manufacturers address device biocompatibility requirements.[12] Part 1 of the norm outlines the rational of tests selection, Part 2 details animal well-being recommendations, and parts 3 through 20 concern specific aspects of biocompatibility test procedures or specific materials test procedures.[13]

Biological studies performed according to ISO 10993 recommendations are acceptable in Europe and most of Asian countries. The FDA has accepted the ISO guideline, but has more strict requirements in some specific areas. In 1995, the FDA published the Blue Book Memorandum G95-1,[14] "Required Biocompatibility Training and Toxicology Profiles for Evaluation of Medical Devices". This regulatory document, as with ISO 10993 guidelines, describes a wide range of biological tests based on the exposure conditions, required in

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order to prove the biological security of their devices under development. In September 2016 this document was superseded by a new guidance for medical device manufacturers and FDA Staff[15] that has the same approach, but emphasizes the risk management process that needs to be implemented for biocompatibility assessment, as it is actually the case in ISO 10993-1. It's useful to mention here that some specific ISO test methods are slightly different from the USP (United States Pharmacopeia) procedures, which were traditionally employed for FDA submissions.[16]

It is noteworthy that these standards represent a general framework to help manufacturers choose suitable tests for biological evaluation, and is not by any means a checklist to fulfill systematically. The manufacturer can avoid performing some (or all) recommended tests for their device, if proof of equivalence (composition, manufacturing process, biological and clinical configurations) to an existing approved device can be made (see annex C of ISO-10993-1).

In the European Medical Devices Directives, it is stated that medical devices should be safe. The essential requirement can be interpreted as the need for a medical device to perform as intended and cause no undue harm. It is, therefore, expected that reasonable measures must be taken to ensure safety. Moreover, biocompatibility studies must comply with Good Laboratory Practice (GLP) regulations regardless of the regulatory procedures used to approve the product.

The framework provided by ISO 10993-1 is completed by the technical report ISO 15499:2012 "Biological evaluation of medical devices – Guidance on the conduct of biological evaluation within a risk management process".[17]

2.1. Case of drug delivery systems and combined medical devices

Biocompatibility requirements compliance also applies to all innovative pharmaceutical entities using drug delivery devices and technologies, including implantable drug delivery devices (such as contraceptive implants, implantable chambers, catheters, implantable infusion pumps), patches for transdermal drug delivery, prefilled syringes and all varieties of drug nanotechnologies (including liposomes, nanoparticles, lipoplexes, dendrimers, micelles, nanoemulsions).

The combination of a drug with a device may help to overcome deficiencies in the device performance related to biocompatibility (for example, devices with heparinized surfaces), prevent anticipated adverse effects (as with antibiotic-containing orthopedic cements) or improves its clinical performance (embolic drug-eluting beads). The product will remain a medical device as the associated drug is intended to have ancillary effect to the main indication of the device itself. Hence biocompatibility evaluations are a mandatory requirement for all of these combination devices. Moreover, the impact of the drug presence on the device interactions with the biological environment has to be investigated during the product preclinical and clinical evaluation.

Even if the distinction between medical devices and medicinal products are at times not obvious, all drug delivery systems, even those considered a pharmaceutical product, can be subjected to biocompatibility tests as part of their toxicological investigation.

During the early stages of development, and prior to animal testing, various inexpensive *in vitro* studies can provide useful information for the initial screening of material safety like cytotoxicity, irritation and hemocompatibility tests. *In vitro* tests offer the opportunity to evaluate materials early in product development to choose the best candidates for future *in vivo* studies, saving time and resources. Those tests can also be used to quality control periodical monitoring on products already on the market permitting production process checking (production or cleaning process toxic residues).

Whereas simple cytotoxicity tests are often used to check biocompatibility of materials, additional assays in areas of inflammation, immunogenicity, mutagenesis and proteomics have the potential to offer complementary information about the biological reaction to materials.

3. Physico-chemistry of biomaterials as a prerequisite for biocompatibility

An early step in medical device manufacturing is the selection of suitable biocompatible materials based on physical and chemical properties. A specific candidate material may have suitable physical properties, but may however be ruled out on the basis of toxicity. Biocompatibility screening includes an assessment of the reaction that cells and tissues may have to a material or to its leachables (such as degradation products, stabilizers, and emulsifiers). Device design and manufacturing processes must minimize enhanced toxicological risks induced by substances leaching from the device.[18]

Moreover, material characterisation data can often serve to reduce the extent of required biological testing. Characterization is crucial to demonstrate the equivalence between the evaluated material/device and a commercialized device. A proof of identical physico-chemical properties and biological application may eliminate the need to perform biocompatibility tests as per the European and the American standards.[10,18]

The device components have to be thoroughly characterized as recommended by the standard ISO 10993- Section 18, chemical characterization of materials[19] before conducting any biological investigations. Data obtained are taken into account when the biological risk is evaluated.

In order to conduct an efficient biocompatibility evaluation, manufacturers need comprehensive knowledge of the device materials, a detailed understanding of the manufacturing process and available information for similar devices. Even if the constitutive materials can clearly be identified, biocompatibility factors may differ significantly following specific processing and finishing steps. This must be considered within biocompatibility evaluation based on a risk analysis and in the change control management.[20,21]

3.1. Physical and mechanical propriety considerations

The local environment in the contact area within the organism governs the mechanical properties required of a biomaterial. Cells are sensitive to their direct environment including chemical signals or morphologic aspects of the surfaces with which they are in contact.

The importance of biomaterial surface properties such as chemical functions, hydrophilicity or hydrophobicity, lubricity, smoothness, surface energy, wettability, surface roughness, swelling, electrostatic effects and protein adsorption in the determination of cells response has been reported elsewhere[22–24] (see below section 5.1). Surfaces properties help to understand cell reactions at a molecular level to direct contact with biomaterials.[10,18,25–27]

Beyond compatibility issues, clinical performance of a medical device depends, for many applications, on the capability to characterise and modify surface properties in a controlled manner.[7,22] Despite the easy manufacturing, reproducibility, degradability and other beneficial properties, polymeric biomaterials lack sharp modular surface properties. By modifying polymeric surfaces (through physical adsorption of molecules and/or chemical modification), it is possible to change surface characteristics and hence interactions with cells. For example, immobilized proteins or other specific molecules can increase polymer hydrophilicity, and decrease unfavourable adhesion phenomenon; micro- or nanopatterning can create structured cellular arrays that influence cell behaviour in contact with material surfaces.[4,6]

Surface wettability, expressed often by the contact angle, significantly influences biological response.[28] Usually hydrophilic surfaces result in a reduced interfacial free energy associated with lower protein adsorption and cell adhesion, as well as an improved hemocompatibility. Increased polymer surface hydrophilicity can be achieved by various techniques such as radiation grafting, plasma discharge and chemical treatments (such as acidic or alkaline treatments). Such surface treatments make the modified polymer more suitable for tissue or cell culture applications.[29] Materials with different water sorption characteristics but equal surface energies may be associated in some circumstances with unmatched biocompatibility profiles through protein and cell adhesion modulations. Moreover, water adsorption and uptake may strongly impact resistance, durability and strength of biomaterials, through hydrolysis-mediated degradation.

When materials are in contact with blood, the adhesion and activation of platelets to biomaterial surfaces are important steps in thrombosis, and are governed in part by surface energy and wettability of the biomaterial surface.[30] Indeed, prior to adhesion of platelets, different plasma proteins adsorb according to the wettability of the biomaterial surface. Adsorption can be modified through the adjustment of surface characteristics, specifically surface energy, of the material.[22,23] If the surface must be non-thrombogenic, as in blood-contact materials, then surface charge and energy are major factors to be considered since these regulate interactions between fluid and materials within the host. Previous studies reported that high charge density resulted in decreased protein adhesion on the surface and better blood compatibility.[28,31]

Roughness of the device surface could have a major impact on bone cell adhesion and consequently on the overall osteoformation.[27,32] However, high surface roughness, achieved by increasing the contact area of the material, could increase macrophage adhesion.[7] It also has been demonstrated that the topography at a nanometric level has an impact on platelet and monocyte activation in addition to cell adhesion and growth on the surface.[33]

Traditional methods for surface characterization include IR Spectroscopy, contact angle measurements, Atomic Force Microscopy (AFM), X-ray Photoelectron Spectroscopy (XPS), Scanning Electron Microscopy (SEM) or Energy-Dispersive X-ray Analysis (EDX). Focal adhesion quantification, recently introduced, may further support the identification of cytocompatible materials and surfaces even if, due to limitations, these results should be interpreted carefully.[34]

Other important physical characteristics of biomaterials include tensile strength, elongation properties, hardness, bonding capability, durability and permeability.[7,10]

In the specific case of nanotechnology, their small size allows nanoparticles to penetrate certain biological barriers and even penetrate cells. Moreover, the high surface area associated with nanoparticles induces a greater contact with biological environment. These characteristics of nanoparticles must be thoroughly evaluated as a biological risk.

3.2. Chemical composition

The chemical composition of a polymer determines key processing possibilities and properties including solubility, degradability, thermal and mechanical stability. Comprehensive chemical characterisation and analysis of the final product, as well as leachate release profiles, are important aspects of biological risk evaluation[1] to which standards ISO 10993-17 and 18,[19,35] are dedicated. Alternative approaches can be used for chemical characterization and risk assessment with the use of computational models to evaluate exposure of extractables and leachables in medical device polymers.[36,37]

Typical extractables and leachables associated with polymers include additives, processing aids and to a lesser extent monomers and oligomers. For example, the chemical employed to achieve radiopacity of polymers is of paramount importance for radiopaque medical devices: Barium salts, used as a radiopacifier, may salt out and cause cytotoxicity, while the polymer itself is not toxic.

The effects of a chemical on patient safety are determined in part, by a measure of human daily exposure (dose of the chemical per body weight).

In addition to raw materials, extractables and leachables testing include any compositional modifications that may take place during the utilization of the device. Those tests should be done using adequate solvents (aqueous and non-aqueous) able to maximize leachable molecules in order to perform biological testing afterwards. Resulting solutions of a simulated

biological environment can also be tested to ensure that leached elements are minimal. Inductively coupled plasma mass spectrometry (ICP-MS) can be used for the identification and quantification of metallic species and GC/LC-MS for molecular species.

As mentioned above, the surface of the biomaterial and the interfacial properties can control the device performance. For example, classical and alternative pathways of complement cascade as well as the consequent recruitment and activation of phagocytes and leukocytes are influenced by surface chemistry.[23]

Additives such as antioxidants could change with time, resulting in a phenomenon of blooming and recrystallization onto polymeric surfaces of medical devices. This could induce wettability lowering, increasing additive leaching into aqueous media[38] and enhanced bacterial adhesion.[39]

Polymer-based biomaterials must also be evaluated with respect to chemical and lipid resistance. Chemicals can indeed affect surface appearance, colour, flexibility, strength, dimensions or polymer weight. Chemical reactions on the polymer chain could impact absorption, permeation of solvents, dissolution or induce stress cracking. Data obtained from material analysis help to characterize toxicological risk or biological effect, but also to demonstrate equivalence of the evaluated material to an already approved reference or to screen potential new materials as seen previously.[19,35]

For the same reasons, chemical resistance to sterilization, shelf life and degradation[40] have to be assessed and must not result in cytotoxic or pro-inflammatory leachable or debris. Hydrolytic and enzymatic stability have to be carefully examined regarding the future use of biomaterials and their interactions with biological fluids.[1]

3.3. Manufacturing considerations

Using biocompatible raw materials should not be considered as a guarantee that the finished device itself is biocompatible. The manufacturing process should be carefully investigated since it could have an impact on the material and its safety profile. In this regard, process variability of every step (machining of bulk, solution/suspension/emulsion polymerization, molding, extruding, fiber forming, *etc*.) must be considered.

For instance, molding process parameters that need to be documented include the duration of the molding cycle, generation of regrind (particles of less than 10 mm in diameter) or the rates of melt flow.[41]

For naturally derived polymers, the development of reproducible production methods is a real challenge, mainly because of structural complexity and variations related to production in living organisms.[7]

Consequences of polymer sterilization, by stem, dry heat, ethylene oxide or various irradiation-based methods, on biocompatibility include, among other impacts, a potential modification of biomaterial integrity, bioresorbability, chemical or pharmacological agent incorporation and generation of leachables or toxic by-products.[42] For example, cyclo-olefin copolymer irradiation by an electron beam (25–150 kGy) resulted in increased surface roughness and a higher wettability with consequences on interactions with drugs.[43] For these reasons, any impact to the final device upon sterilization must be assessed.

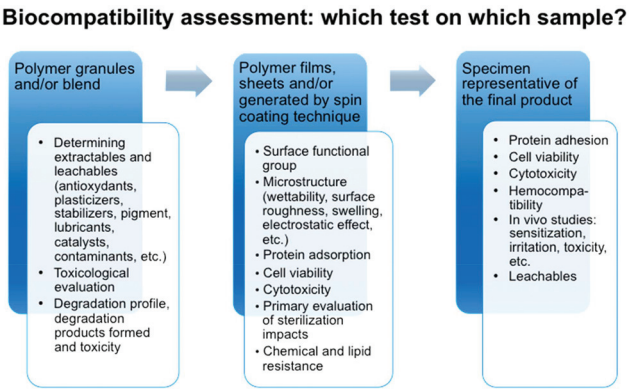

Fig. 1 Assessment/evaluation plan according to present knowledge of polymers.

As is the case with biological *in vitro* testing, chemical and physical characterization of biomaterials can be used to justify certain animal tests based on scientific judgment and expert analysis, and are necessary for the communication with regulatory authorities (Fig. 1).

The main material parameters impacting host interactions and needing to be controlled for polymers include the following:[2]

- Bulk properties: composition, porosity, crystallinity, and water content elastic constants.

- Surface properties: topography, chemical composition, energy, molecular mobility, electrical properties and hydrophobic/hydrophilic balance.

- Degradation: kinetics, products and toxicity.

- Leachables including additives, debris, contaminant and associated toxicity.

4. Evaluation based on risk assessment and medical device use

The safety of all devices entering into contact with the human body must to be evaluated within the context of a risk management process. Hazard identification is an essential step in ensuring safety.

Safety evaluation in relation to toxicity can be performed following the ISO 10993 series.[13] This series features a review of the existing method knowledge, with the rationale for selection and implementation of additional experiments to cover biological safety aspects that were not previously elucidated. However, such standards are designed for use with discernment by knowledgeable and experienced professionals who have the ability to judge the suitability of their application. ISO 10993-1 places biological testing in its proper context and points out the importance of other relevant aspects of an assessment.[18] Tests suggested within EN/ISO 10993-1 should

be considered neither as a required check list nor an exhaustive one. In fact, the normative requirements recommend that the rationale for conducting a test or not is recorded.[5,17]

During product reviews, both European authorities and FDA accept combined data from literature/clinical history and limited biological investigations, and does not encourage companies to conduct unnecessary testing. In this case, documentation of all materials used in the device, user and/or patient contact routes and duration, history of similar device use and specific production processes likely to affect the material must be provided.[32,41]

In order to confirm that the test plan is appropriate, it is possible (and even recommended) to discuss it with regulation authorities (Fig. 2).

ISO 14971 standard, "Application of risk management to medical devices", is intended to provide a framework to manufacturers in which knowledge, understanding and scientific judgement are applied to assess and control the risks related to the use of medical devices.[44] This standard, applicable to all life-cycle stages of the product, is a guideline on how to identify, estimate and manage risks associated with the use of the device as well as the evaluation of residual risk.

The technical report ISO 15499:2012[17] also gives some illustrations on how biological evaluation of a biomaterial or a medical device should be processed within a risk management approach and presents ways that could be used to meet requirements of ISO 10993-1 and ISO 14971.

Before determining if a product made from a specific material is suitable for its intended use, physico-chemical and manufacturing factors that may affect the appropriateness of sample to the considered application should be taken into account in order to make, if necessary, additional specific tests. The design of tests and the preparation of samples or extracts must take into account hazards and risks estimated for medical devices as well as the hazards that arise from manufacturing process modifications or insufficient controls.[17,45]

Potential biological hazards have a wide range of effects from short-term risks including irritation, thrombose, haemolysis, acute systemic toxicity, to longer term risks such as genotoxicity (*i.e.* the potential of inducing damages of the genetic information within a cell causing mutations) or carcinogeni-

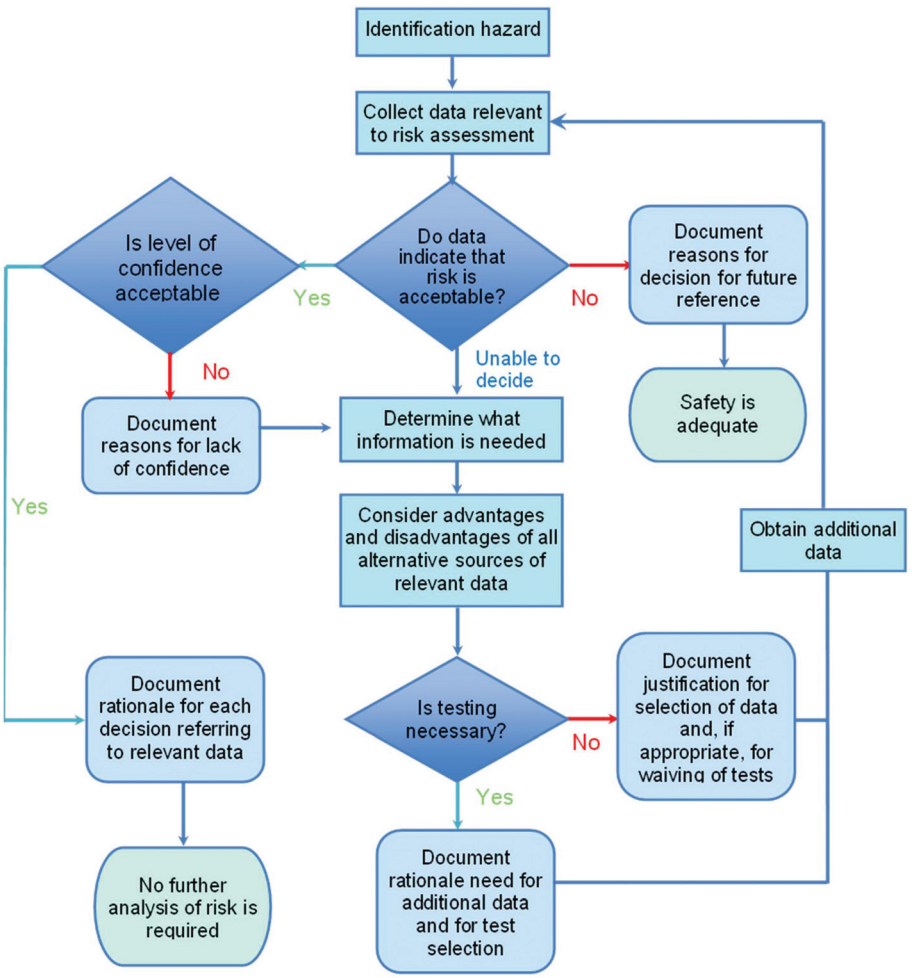

Fig. 2 Rational approach for documentation of testing in biocompatibility evaluation (ISO 10993-1[12], ISO 14971[41], ISO 15499[15]).

city (*i.e.* the ability to induce the formation of cancer and promote the growth of malignant cells) among others.[20,21]

Both *in vitro* and *in vivo* investigations of biocompatibility are chosen to evaluate the safety of the final product. To optimally standardize appropriate test choices, medical devices were categorized according to the duration and the level of tissue contact (from surface contact to implantation) (Fig. 3).

Although the evaluation of the final product is strongly recommended, device material testing using chemical characterization as suggested by ISO 15499:2012[17] may help to improve biological risk analysis in the early stages of device development, and consequently reduce biocompatibility related failures of the final product. Any change in the design of the biomaterial must result in a re-evaluation of the risks previously assessed, in order to be sure that biological performance of the device is not altered. The effect of any change in the process has to be evaluated and complementary tests may be necessary to help reach a conclusion.[17]

Greater test sensitivity leads to increased reliability of identifying hazards, but reduces specificity at characterising risks.[46]

Biocompatibility should not be considered as a given property of any material but rather it depends on the biologic environment and the latitude that exists for tissue reaction.[25] In fact, medical grade materials (or surgical or implantable grade) do not exist and there is no regulation or standard that defines what a medical grade material would be. For instance, implantation success in the orthopaedic field will need quite different investigations than a vascular stent.[9]

Only limited biological evaluation tests, including cytotoxicity, sensitization and implantation, may be required for devices produced with materials whose safety data are long-lasting established. These tests are efficient tools to investigate material reactivity changes following production process modifications, leading to deeper investigations if necessary. Obviously, for particular routes and exposure durations of

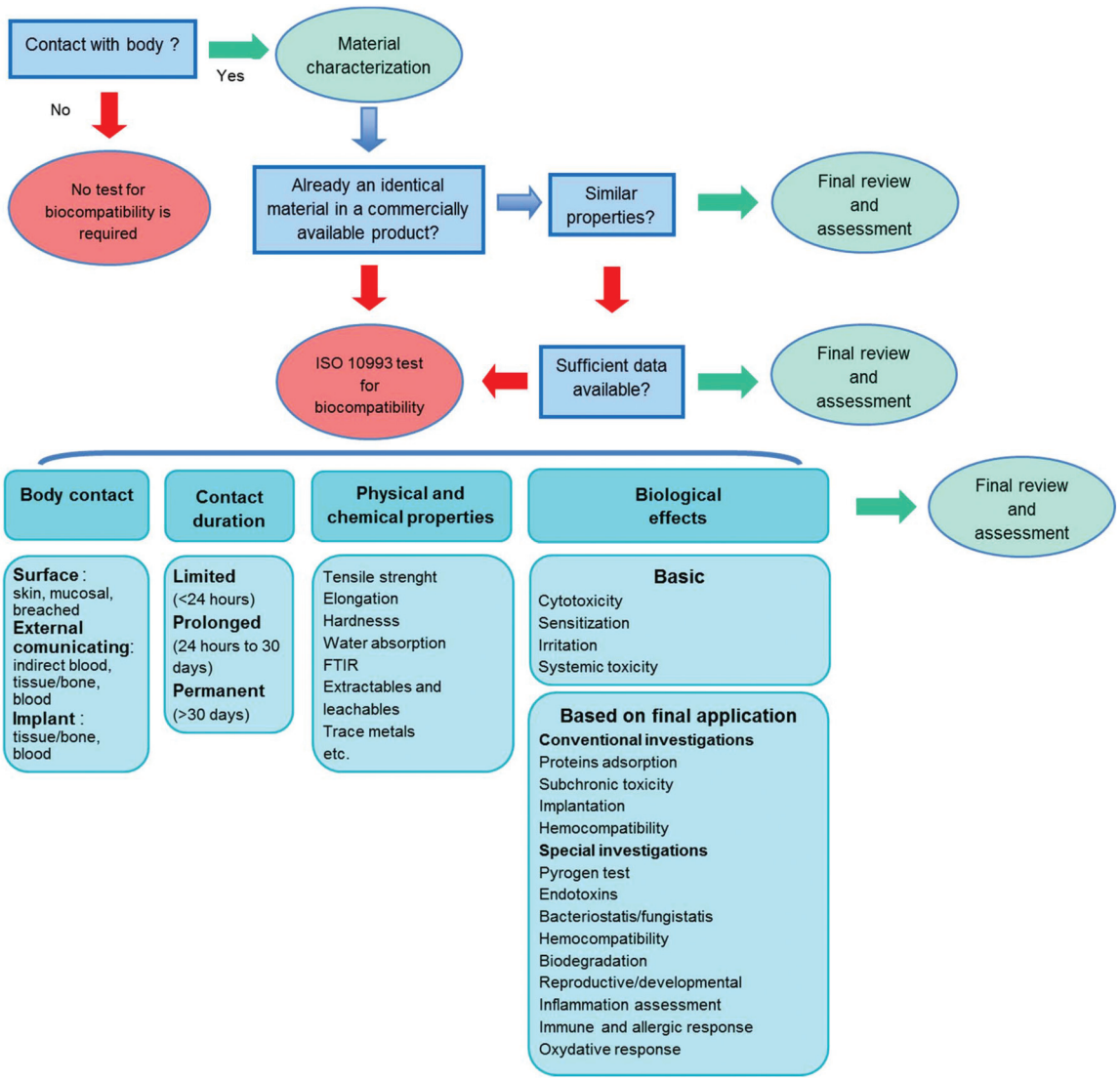

Fig. 3 Decisional tree according to medical device use, short/long term.

some materials, systemic toxicity, genotoxicity and carcinogenicity have to be assessed with appropriate investigations. However, to reduce the need for formal carcinogenicity testing, it can be argued that safety data for the device materials are already established associated with negative genotoxicity results.[17]

To establish a biocompatibility test plan, a number of steps have to be followed[4,17,20,21,32,41] (Fig. 4):

- Documentation of the complete description of the device (including packaging components known to contain toxic residues), generic chemical identify of the materials used, contact tissue and duration, population of patients (paediatric, *etc*.).

Exposure duration categories are defined as limited (<24 h), prolonged (24 h–30 days) and permanent (>30 days). If a high likelihood of retreatment exists, multiple device exposures must be incorporated into the biocompatibility-testing plan.

Routes of exposure are conventionally classified as (i) external contact (skin, mucosal membrane or compromised surfaces), (ii) external communicating (contact with blood, tissue/bone/dentin or circulating blood) and (iii) implant devices (in tissue, bone or blood).

- Documentation of all pertinent data about component materials and available data from similar devices with long history of safe use (see above).

- Exposure assessment with information on the composition of biomaterial: formulations, residue levels, degradation products, effects of processing, *etc*. in order to characterise any toxicological risks. Quantitative data on levels of ingredients, residues or on the amounts leached from the material allow estimation to the likelihood of adverse effects arising from exposure to specific hazards that have been identified.

- Based on a biological risk analysis, material specifications can be defined, like residue limits or critical material attributes for example. *In vitro* tests can be performed on components and/or on the final product.

- Carry out *in vivo* biological investigations on the finished product to demonstrate the absence of unacceptably harmful effects.

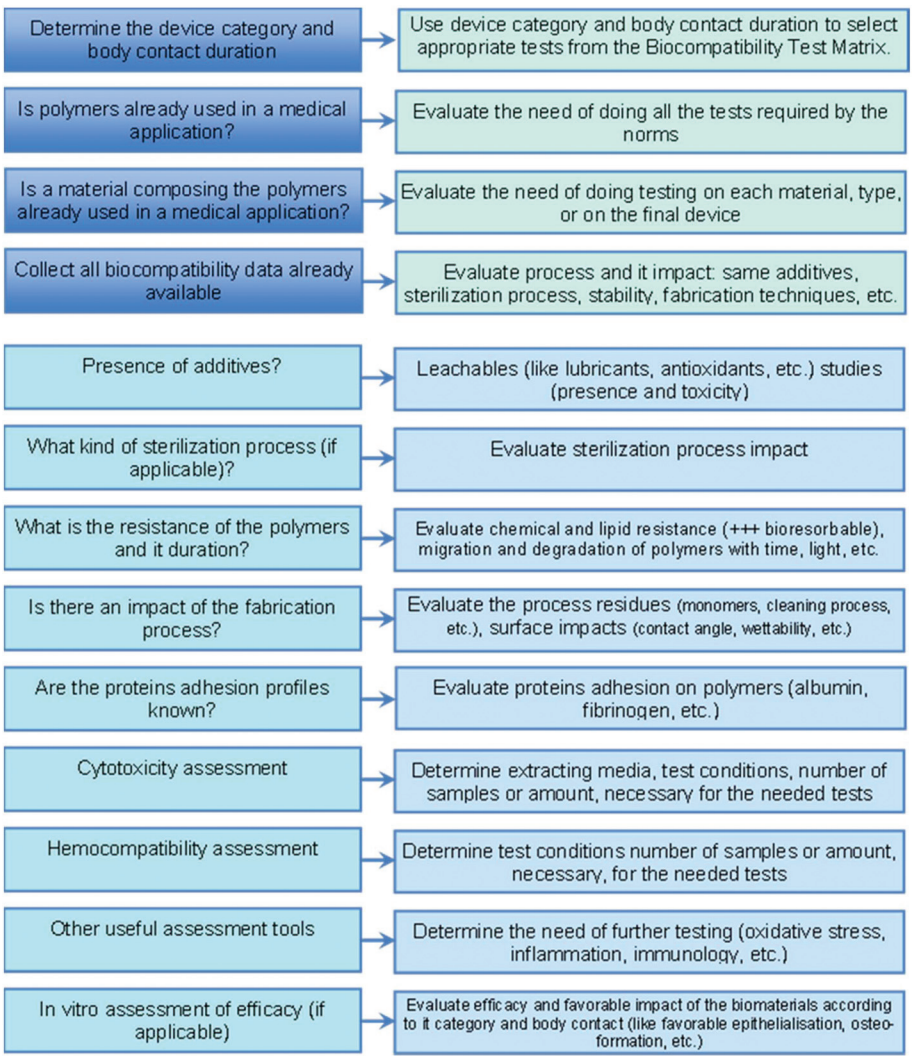

Fig. 4 Example of how to proceed for the elaboration of an *in vitro* biocompatibility-testing program.

It must be kept in mind that the host response, involving both humoral and cellular components, is extremely complex and often involve amplification or cascade events. There is often a two-way relationship between the material and host response (degradation process is pro-inflammatory and the products of inflammation enhance the degradation). The mechanical stability influences the host response, and in many situations the host response determines the stability. It is important to highlight the fact that the host response is time dependent, patient specific (depending on age, sex, health status/concomitant disease, pharmacological status, lifestyle, *etc*.) and biocompatibility is species specific.[2,4]

4.1. Questions of quality assurance and reference materials

Biocompatibility testing must be performed with respect to Good Laboratory Practice (GLP) as required by FDA and ISO 15499:2012.[17] GLP guidelines provide the essential requirements for planning, performing and documentation of laboratory studies, in order to guarantee that results from nonclinical studies could be verified, would be repeatable and would produce scientifically valid data.[17,20,21]

In many biocompatibility tests, reference materials are used to serve as experimental controls. A negative control is a material that is known to comply with the executed test; while the blank, used mainly with tests implicating liquid samples, is composed exclusively from the solvent used to prepare the investigated liquid samples. Standard 10993-5[47] specifies a high-density polyethylene, obtained from U.S. Pharmacopoeia, as a negative control and organotin-stabilized polyurethane as a positive control (ZDEC and ZDBC polyurethans). Polyvinyl chloride with organotin additives can also be used as a positive control. Moreover, controls should be selected so that they can be prepared by the same procedure as the test sample.[48]

In the following sections, some examples of risk management approaches are given according to the body contact, knowing that detailed *in vitro* methods are described later (see section 5.). ISO 10993-1:2009 and new FDA guidance provide a matrix of evaluation tests that constitute a framework for biological effect assessment and could easily be used for planning.[12,15]

4.2. Skin contact

Devices that are in contact with healthy skin include patches, external prostheses, and various kinds of bandages, monitors, electrodes and tapes. Cytotoxicity, sensitization and irritation testing are usually required to evaluate their biocompatibility. Sensitization tests allow investigation of the risk of allergic responses to the device and/or their leachates. These tests are generally performed in rabbits or Guinea pigs using pertinent route and exposure conditions. The use of biomaterial extracts determines the irritant effects of potential leachates and must be carefully selected in order to have physiological pertinence.[1]

4.3. Mucosa contact

Devices in contact with mucosal membranes, such as urinary catheters, contact lenses, intravaginal and intraintestinal devices, bronchoscopes, endotracheal tubes, mucoadhesive devices for oral/ocular/rectal/vaginal drug administration, dental prostheses and orthodontic devices require toxicity assessments according to the contact duration. Systemic, subchronic toxicity, genotoxicity and chronic toxicity must be considered as well as pyrogenicity testing (part of the systemic or acute toxicity category).

4.4. Blood contact

Devices in contact with blood or used to access the circulation include those intended for perfusion, solution administration, extension, transfer or blood administration. Hemocompatibility studies are required and described in ISO 10933-4 "Selection of tests for interactions with blood".[49]

We have seen previously that plasma protein adsorption and platelet adhesion followed by activation may result in blood coagulation and consequently to device failure due to thrombus formation. Thus, disruption of the blood cells (haemolysis), thrombogenicity and/or the activation of complement proteins must be assessed.

For long-term implantation, other factors such as endothelialization phenomenon may also have an important impact on biocompatibility.[50]

4.5. Implant devices

This category of devices is large and encompasses those principally contacting bone, tissue and fluids including blood.

Additional necessary tests include implantation and in certain cases, carcinogenicity evaluation. Implant studies are conducted to investigate the local tissue reaction in direct contact with the final device for a period relevant to the intended clinical conditions. After healing, the device is explanted and the tissue reaction is assessed in terms of fibrous cap formation, necrosis, inflammatory cells distribution and the presence of device degradation products.[11,51] The cellular and immunologic responses or biochemical exchange can be closely monitored using histopathological analysis at implant sites.[1]

Complete blood compatibility evaluation of implants begins first with direct blood contact, especially in the initial phase of implantation (endovascular stents, heart valves, haemodialysis membranes or pacemaker components). Unlike physiological or clinical conditions, anticoagulants are often used during *in vitro* hemocompatibility assessment. *In vivo* hemocompatibility studies, conducted in combination with implantation and/or systemic toxicity, are crucial for thrombosis and vascular tissue reaction assessment. Hemocompatibility data need to be interpreted with caution because of the different blood reactivity of animal species compared to human blood.

The potential of an implanted device to incite the growth of malignant cells is assessed through a carcinogenicity test, especially for permanent contact devices. This risk from either single or multiple exposures has to be investigated *in vivo* only if available data indicate a potential risk of cancerogenesis. A study period over the majority portion of the test animal life-

span is recommended, which is time-consuming and expensive.[52]

Studies on reproductive toxicity are required for permanent contact devices and those containing a source of energy. These *in vivo* tests enable evaluators to obtain information about any potential risks of the device or its extracts on reproductive function, embryonic development (teratogenicity), pre- and postnatal development.

5. Considerations for a rational approach of *in vitro* methods

Biological safety and biocompatibility are often used synonymously but are in fact different concepts. Biological safety is an absence of risk, while biocompatibility is a more positive concept in which an appropriate dynamic equilibrium exists between tissues and biomaterials to address a therapeutic need. Biocompatibility is indeed the "ability of a material to perform with an appropriate host in a specific application".[2,8]

Through simulated clinical exposure conditions, preclinical biocompatibility tests performed on medical devices are intended to investigate potential risks of adverse effects. These tests should be used to complement material characterisation assessments.

In the following sections, the main categories of *in vitro* concepts employed for biocompatibility screening of polymers are reviewed. For each category, the physiological response to biomaterial contact is resumed shortly before the description of experimental methods used for the biological evaluation of medical devices as a part of a global rational approach.

5.1. Proteins interaction and adsorption on the polymer

Adsorbed proteins influence the interactions that occur at the tissue-implant interface.[53,54] Protein adsorption (mainly albumin, Immunoglobulin G, fibrinogen, fibronectin and von Willebrand factor) occurs rapidly (minutes to hours) after implantation onto polymer surfaces followed by cellular interaction with the protein layer (few nanometres of thickness) and not with the device surface itself.[23] During the adsorption process, proteins may undergo conformational changes as discussed in details by Ballet *et al.*[55] The role of protein–surface interactions in the biocompatibility of materials are reviewed elsewhere.[56,57]

Those interactions of proteins on surfaces, named Vroman effect, depend on the protein particularity (size, charge, conformation, unfolding rate) and physico-chemical properties of the materials that influence the adjacent interfacial behaviour (surface chemistry, energy, charge, topography, dissolution rate, elasticity).[54]

Protein adsorption can activate the host foreign/body response: by the complement pathway, neutrophils and macrophages attach to the device surface and lead to its attack by destructive enzymes, superoxide anions and hydrogen peroxide. Bacterial colonization on the implant surface is also promoted by protein adsorption.

Since the amount of spontaneously adsorbed protein is a property of surface chemistry, this could be used as a rapid screening tool at an early stage of development of biomaterials to compare polymers.[24,31,58]

Adsorption profiles (qualitatively and quantitatively) are the major determinants of protein-mediated cell responses and protein bioactivity. Controlling the amount and composition of adsorbed protein, and the degree of protein conformational changes with pro-inflammatory epitope exposure, is the great challenge for modified biomaterial surfaces.[23,30]

Depending to the clinical application, an increase or decrease in surface protein adsorption on the medical device may be desired. For example, it is advantageous to use biomaterials for medical devices that are in contact with the circulatory system to have surface properties associated with low levels of nonspecific protein adsorption. Medical implants can be polymer-coated to reduce protein adsorption, which can cause inflammation or thrombosis. Whatever the case, protein adsorption profiles need to be assessed and optimized. The literature is rich with reports about functionalized surfaces designed to reduce nonspecific protein adsorption. Examples include PEO, PEG (hydrophilic molecules that repel protein adsorption), and phosphoryl choline modified polymer surfaces.[56,57] These coatings reduce protein adsorption on the device surfaces and thus modify fibroblast and bacteria adhesion.[59]

In other cases, such as osteointegration into bioengineered scaffold, interactions between biomaterial surfaces and proteins promote the integration of the device into the biological environment.[9] The improved adsorption of fibronectin and vitronectin on the surface of a bone substitute material[60] and nanofibrous scaffolds[61] were reported to enhance osteoblast attachment and proliferation.

For a blood contacting material, thrombosis and embolism remain major challenges. Plasma protein adsorption onto polymer surfaces can induce biochemical reactions potentially leading to blood clotting. Factor XII is known to be activated by negatively charged surfaces and may result in blood coagulation through induction of the intrinsic pathway.[62] In the case of thrombogenesis control, materials that do not induce excessive platelet adhesion and activation through plasma protein adsorption or blood compounds are generally non-thrombogenic.[3] The interaction between platelet and blood contacting devices is mediated by the presence of adhesion receptors on platelet membrane that recognise specific plasma proteins adsorbed on the device surface.

Shortly after implantation, thrombogenic surfaces are subjected to the adsorption of fibrinogen, a glycoprotein found in human plasma at a concentration of around 3 mg mL^{-1}. Its conversion from soluble fibrinogen into the insoluble fibrin leads to blood clotting. It is well documented that fibrin plays an important role in neovascularization, angiogenesis as well as platelet adhesion.[58,63–65] Under flow conditions, fibrinogen and von Willebrand factor mediate platelet adhesion.[66] It was reported that the expression of fibrinogen active regions varies depending on polymers.[67] Fibronectin also enhances cell

adhesion and spreading, platelet attachment and aggregation.[68] Thus a surface that could limit protein adsorption is expected to have improved hemocompatibility.

Taken together, the above-mentioned issues highlight the importance of studying protein adhesion to biomaterials towards the evaluation of hemocompatibility, and more broadly biocompatibility.

Proteins in solution can easily be quantified using ultraviolet-visible spectroscopy and conventional colorimetric methods such as BCA, Bradford, or Lowry based assays. Also enzyme linked immunosorbent assay (ELISA) is employed to identify and quantify proteins in solution. However, adsorption kinetics and protein conformation cannot be assessed with these methods.

Adsorbed protein detection is generally more complicated as they are associated with a solid substrate. Different methods are now available to detect, identify and quantify adsorbed proteins, where each one has its own advantages, constraints and drawbacks. The choice of method will depend mainly on the specific information required (quantification of a specific protein from a mixture of proteins, protein conformation, adsorption kinetics, molecular visualization or the topography of the adsorbed layers). Most often a multiple approaches are required to obtain the needed information.

When protein adsorption dynamics and kinetics are of interest, surface plasmon resonance (SPR), Quartz Crystal Microbalance with Dissipation (QCM-D), Ellipsometry and atomic force microscopy (AFM) are valuable techniques. Detection, localization and quantification of a specific protein within a mixture adsorbed on a substrate is possible using ELISA. Experimental tools to investigate protein conformation include IR for bond remodeling assessment, QCM-D, SPR and ELISA employing conformation sensitive antibodies. AFM can resolve internal macromolecular movements with a high resolution, and give valuable information about adhesion forces and surface topography.[55]

Principles, analytical sensitivity, special resolution and information obtained from the most employed methods for proteins–biomaterials interactions investigation have been summarized by Martins *et al.*[69]

Examples of these techniques used for protein adsorption studies on various types of materials are presented in Table 1.

5.2. Choice of cells and cellular model for *in vitro* tests

Cell-culture based techniques employed for biological evaluation aim to investigate the biological responses at a cellular level in models that simulate, as possible, the *in vivo* environment. These techniques serve also in some cases to evaluate the functional performance of the studied biomaterials (Fig. 5).

The choice of cell lines used for cytocompatibility assessment must be carefully discussed beyond all regulatory considerations. In most cases, continuous (immortalized) cell lines originally obtained from neoplasms or transformed by viruses or mutagens, such as HeLa, L929, WI-38, 3T3 or CHO, are used in the screening stage of cytotoxicity testing, when common cellular endpoints are investigated.[50] In a second step of cytocompatibility evaluation, the cells are chosen according to interactions investigated and prospective medical applications. Human or animal cells such as immune, endothelial, hepatic cells or various epithelial cell lines can be used.[1,46,82,83] For instance, it is crucial to evaluate endothelial cell and blood platelet reactions for devices in contact with the vascular system, since they will determine implant success through their specific factors.[1] For skin contact materials, the use of fibroblasts such as mouse fibroblast L929 cells is relevant and adequate, since they are reported to give reproducible results and good correlation with animal-based tests, in addition to their physiological role in the wound healing process around implanted devices. In dentistry or odontology, odontoblasts, gingival fibroblasts and periodontal ligament cells are widely used cell lines to evaluate cytotoxicity of devices.[83] For cell-based assays to evaluate contact lens or their care solutions, the use of human conjunctival or corneal epithelial cells seems more appropriate as discussed by Dutot *et al.*[84,85] Human lens epithelial cells are the preferred model for the studies of interactions with intraocular lens.[75]

5.3. Sample preparation and the issue of direct contact, indirect contact and extract methods

Sample preparation for biocompatibility testing is dealt with ISO 10993-12 "Sample Preparation and Reference Materials" in additions to the methods of USP or ASTM.[48]

Test samples are put in contact with cells whose viability/proliferation/degeneration/malformation/lysis are evaluated 24–72 h later. Three contact methods between cells and devices are suggested by international standards:[47] (i) direct contact test, (ii) indirect contact through a layer of agar or a filter diffusion test, (iii) extract methods based on the application of extraction media (under physiological or exaggerated conditions) onto cells before cytotoxicity evaluation.

The direct contact method is intended to evaluate cytotoxicity of the intact device; meanwhile indirect contact techniques allow the investigator to detect leachable substances that could exert toxic effects. For novel materials, direct contact and elution methods are both recommended.

It is recommended that extraction conditions should be chosen to obtain the maximum of extractables and simulate extreme conditions to which the test device or material may be exposed, but without inducing material degradation.[50] The choice of extraction medium, temperature and time should be relevant to the nature of the finished product, purpose of the test, and the physicochemical properties of the materials composing the device and any other known leachable substances or residues. A variety of polar and non-polar solvents as well as a detailed recommendation on material surface or weight-to-solvent-volume ratio can be found in the standard ISO 10993-12. Extracts should not be manipulated by filtration or centrifugation to avoid particles or chemical retrieval.

In the case of solid samples, the physical shape may impact *in vitro* and *in vivo* data.[46,50] Usually the most convenient shape of the material sample for the experiments is selected

Table 1 Common techniques used for the study of protein adsorption on different types of material surfaces

Techniques	Materials	Proteins	Information investigated	References
Direct dosage UV absorbance (λ = 280 nm)	PU films	BSA, BSF	Quantification – correlation with surface roughness wettability and swelling	Akkas (2013)[22]
	Hydrophilic surface (silicon oxide) *vs.* hydrophobic methylated surface	BSA, lysozyme, α-synuclein	Interplay between protein and surface	Ouberai (2014)[31]
Dosage in UV at 202 and 210 nm after desorption	PU (PEU, PCU)	BSA, BSF	Interplay between protein and surface Correlation with adhesion and proliferation of cells (HUVEC, platelet, monocytes)	Hsu (2004)[70]
Radiolabeled proteins	Tetraglyme coated surfaces	Fg (^{125}I), vWf (^{125}I)	Quantification – correlation with hemocompatibility	Cao (2007)[71]
	PEO modified PU	Fg (^{125}I), concanavalin A (^{125}I), myoglobin (^{125}I), albumin (^{125}I), ferritin (^{125}I)	Quantification – correlation with PEO grafted length, protein size, and protein charge	Archambault (2004)[72]
	Nanofibrous PTFE	BSA (^{125}I)	Quantification	Ainslie (2007)[73]
FITC labeled protein	PDMS coated with zwitterionic moities	FITC BSA	Quantities – correlation with antifouling properties	Diaz Blanco (2014)[74]
ELISA	Hydrophobic polyarcylates Intraoccular implantes	Fn	Quantification and correlation with materials aging	Tortolano (2015)[75]
	Titanium substrate (heparin/Fn films on aminosilanized titanium surfaces)	Fn	Quantification of conjugated fibronectin – correlation with hemocompatibility	Li (2011)[76]
SPR	Tetraglyme coated surfaces	Dilutions of human plasma	Protein adsorption kinetics from different plasma concentrations	Cao (2007)[71]
Biotin conjugated proteins	PU films	Fn, Fb, collagen I, collagen II	Quantification – correlation with fibroblasts and platelets adhesion	Faré (2005)[77]
Ellipsometry, QCM-D, OWLS	Titanium oxide surfaces	Human serum albumin, Fg and haemoglobin	Quantification – competitive protein adsorption, kinetics	Höök (2002)[78]
QCM-D	Gold coated with hydrophilic or hydrophilic groups	BSA, BSF	Binding rates amounts	Roach (2005)[79]
FTIR	Gold coated with hydrophilic or hydrophilic groups	BSA, BSF	Protein conformation	Roach (2005)[79]
AFM	Chemically modified silicon surfaces	BSA, collagen	Quantification –competitive protein adsorption	Ying (2003)[80]
	Methyl and amine functionalized gold surfaces	BSA	Conformation	Taborelli (1995)[81]
SDS-PAGE followed by liquid chromatography MS/MS	TiN coated NiTi alloys	Culture medium supplemented with 20% bovine serum	Quantification – mediation of proteins to cell adhesion and growth	Yang (2013)[82]

AFM: atomic force microscopy, BSA: bovine serum albumin, BSF: bovine serum fibrinogen, FITC-BSA: Fluorescein isothiocyanate labelled bovine serum albumin, Fg: fibrinogen, Fn: fibronectin, FTIR: Fourier transform infrared spectroscopy, PDMS: polydimethylsiloxane; PEO: poly(ethylene oxide), PTFE: polytetrafluoroethylene, PU: polyurethanes, OWLS: optical waveguide lightmode spectroscopy, QCM-D: quartz crystal microbalance with dissipation monitoring, SPR: surface plasmon resonance, TiN coated NiTi: titanium nitride coated nickel–titanium alloys, vWf: Von Willebrand factor.

but it is often different from the final product. This is also an issue to keep in mind in the risk analysis process.

5.4. Cytotoxicity

Cytotoxicity investigations using cell culture-based methods are performed at the first step of screening of material biocompatibility.[41] They are sensitive, reliable, convenient and reproducible despite a lack of specificity.[86,87] Cytotoxicity assays provide qualitative and quantitative estimations of the potential danger of a biomaterial. A positive result must be taken as an early warning sign of a potential biological risk of the material itself, requiring further investigations. A combination of different assays can provide a more comprehensive determination of the cytotoxicity mechanism resulting in cell death.

Cytotoxicity can be measured by a variety of literature methods, and therefore it is crucial to clearly specify assay conditions (cell type, number, phase and exposure details) in order to compare and correctly interpret results.

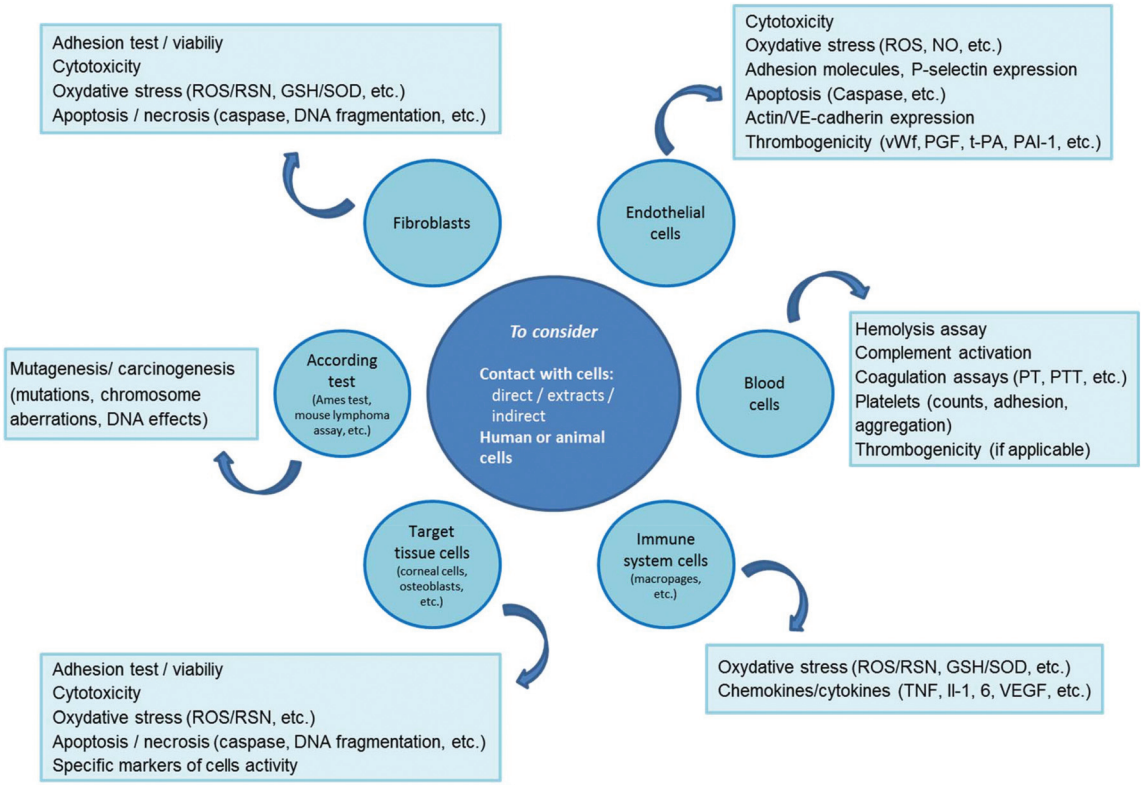

Fig. 5 *In vitro* tests for biocompatibility assessment and conventional cell model choice. ROS: reactive oxygen species, RSN: reactive nitrogen species, GSH: glutathione, SOD: superoxide dismutase, vWf: von Willebrand factor, PGF: prostaglandin F, t-PA: plasminogen tissue activator, PAI-1: plasminogen activator inhibitor, PT: prothrombin time, PTT: partial thromboplastin time assay.

Besides morphological investigations based on microscopic examination of cells, viability tests are based on the measurement of a wide variety of cellular functions (Table 2). The most commonly used assays are presented herein.

Cell counting. Counting the number of viable/dead cells using a cytometer (such as Malassez and Neubauer chambers) or automatic cell counters is a basic and simple starting point for cytotoxicity evaluation. In the Colony Formation method, the number of colonies (commonly V79 cells) that grow in contact with a test sample are compared microscopically to those of a control.[88,89]

Vital dyes. Vital dyes (or stains) are small molecules that interact with cells based on cell state and integrity. Trypan blue (dye exclusion method) can enter inside cells when they are damaged or dead, and then, can be counted with a cytometer. In the neutral red uptake (NRU)-based assay cells capable of incorporating the dye into their lysosomes are labeled viable.

Biochemical based assays. These tests are less subject to analyst interpretation than vital dyes tests, and could be used more reliably for dose/response studies.[86,90,91] They are the most widely used viability assays reported in the scientific literature. Formazan-based methods are used to assess cell viability and proliferation, and include MTT, MTS, XTT, INT and WST-1 colorimetric assays that measure the mitochondrial activity of dehydrogenases in viable cells. The relative cell viability can be quantified in comparison to controls. These methods do not discriminate between specific cellular death mechanisms. Moreover, as these methods are based on the quantification of living *versus* dead cells, toxicity mechanisms that do not result in cell death may be underestimated. The lactate dehydrogenase (LDH) release assay is widely used as a measure of cell viability, since cellular membrane damage results in the liberation of this cytosolic enzyme and the increase of its extracellular concentration. Cell proliferation measurement using the ELISA BrdU (bromodeoxyuridine) colorimetric immunoassay is based on the quantification of cell-associated reagent during DNA replication. This technique can replace the radioactive (^{3}H)-thymidine bioassay (see below). The sulforhodamine b (srb) assay binds cell proteins and the intensity of its absorbance can be then used to measure cell density in comparison with controls.[92]

Fluorescence-based assays. Intracellular nonspecific esterase-mediated hydrolysis of calcein acetoxymethyl, a non-fluorescent conjugate, results in the release of the green fluorescent calcein inside both healthy and damaged cells. Hence its combination with other fluorescent probes, such as propedium iodide or 7-aminoactinomycin D that can penetrate only damaged cells, may allow determining the ratio of healthy/damages cells.

Radioactivity-based assays. These techniques employ radioactive elements for the quantification of cell damage.

Table 2 Classical cell viability assays in polymers biocompatibility assessment

Techniques	Materials	Cell lines	Information investigated	References
Morphological means	Glass PU Glass + chitosan Glass + hyaluronic acid	ThP1	Correlation of macrophage morphology and inflammatory responses (cytokines)	Lee (2013)[98]
Morphological means	Corn starch with ethylene vinyl alcohol ± hydroxyapatite	Human osteosarcoma cells	Cell adhesion and proliferation on the materials surface	Gomes (2001)[99]
Morphological means	PU (PEU, PCU)	HUVEC, human bood monocytes	Cell adhesion and proliferation on the materials surface	Hsu (2004)[70]
Morphological means	Copolymers of PCL and PEG	HUVEC	Counting, immunofluorescene and cytoskeleton stain	Hsu (2004)[33]
Morphological means, LDH, MTT	PCL	L929	Adhesion, proliferation, cell viability, mitochondrial activity	Serrano (2004)[100]
Trypan blue dye exclusion method	UDMA-MAA	3T3 fibrobalst	Numbers of stain ± cells Comparison between different copolymer's composition	Cao (2011)[101]
NRU	PVP, PEG	L929	Biocompatibility of polymeric materials used in contact with skin	Rogero (2003)[102]
NRU (membrane integrity) Alamar Blue assay (metabolic activity) MTT	Poloxamer, poloxamine contained in contact lens Multipurpose solutions	Conjonctival epithelial cells, human corneal epithelial cells	Use of neutral red and Alamar Blue assays for the evaluation of ocular medical devices, adaptation of ISO 10993-5 standard	Dutot (2012)[85]
→Direct contact →Agar diffusion →Extracts Morphological, vital stains, cells proliferation	Metals, PU, PVC, PET, PDMS, PEHD, PTFE, PMM, fluorocarbon	L929, NBL-6/8/9/12, Vero, Neuro-2a, Girardi heart, chang liver, WI-38, Citrullinemia, CCL 20.2	Comparison between techniques, cell's model and *in vivo*/*vitro*	Johnson (1983)[46]
Morphological means and proliferation by fluorescence and electron microscopy, MTT	CNT-PEDOT-coated electrodes	NB-39-Nu human neuroblastoma cells	Biocompatibility linked to chemical and mechanical features, promotion of neuronal adhesion and outgrowth	Depan (2014)[103]
Morphological means, MTT, apoptosis (*via* PS-annexin V) and proliferation (*via* fluorescent dye dilution using flow cytometry)	PAN/carbon nanofibers or thin films	Schwan cells	Relation between cell proliferation, metabolic activity and apoptosis and time dependent evolution of intracellular oxidative stress. Carbon substrates toxicity assessment on neural cells	Jain (2013)[104]
WST	TPU	CCL-110	Impact of steam sterilization	Haugen (2006)[105]
Calcein AM for live cell staining *vs.* ethidium homodimer-1 for dead cell staining Trypan blue for cell viability assessment	PEG, PDMS, parylene-C	NIH3T3 fibroblasts	*In vitro vs. in vivo* cytotoxicity comparison of 3 materials	Su-Jin (2012)[106]
Focal adhesion (b-Tubulin III, F-actin and vinculin staining)	Parylene C, silicon oxide	Mouse embryonic stem cell CGR8	Role of serum protein adsorbed (albumin, fibronectin) on polymer on cell adhesion	Delivopoulos (2015)[59]
Alamar blue based assay (CellQuanti-Blue™) Morphological by confocal laser scanning and SEM	PLLA, PDLLA, P(LLA-*co*-GA), P(DLLA-*co*-GA), P(LLA-*co*-CL)	L929, HCAEC (human coronary artery endothelial cells), HUVEC	Biocompatibility evaluation of biodegradable polymer materials and influence of surface modification (plasma treatment)	Rudolph (2015)[107]
LDH and blue trypan counting, with a Transwell-chamber method (migratory response)	PU films	Human skin and gingival fibroblasts	Adhesion and proliferation of fibroblasts, correlation with protein adsorption and platelets adhesion	Faré (2005)[77]

CNT-PEDOT: carbon nanotubes coated with poly(3,4-ethylenedioxythiophene), MAA: methacrylic acid, PAN: polyacrylonitrile, PDMS: polydimethylsiloxane; PCL: poly(e-caprolactone), PCU: poly(carbonate)urethane, PDMS: polydimethylsiloxane, PEG: poly(ethylene glycol), PEHD: polyethylene high density, PET: poly(ethyleneterephtalate), PEU: poly(ether)urethane, PEO: poly(ethylene oxide), PLLA: poly(L-lactide), PDLLA: poly(D,L-lactide), P(LLA-*co*-GA): poly(L-lactide-*co*-glycolide), P(DLLA-*co*-GA): poly(D,L-lactide-*co*-glycolide), P(LLA-*co*-CL): poly(L-lactide-*co*-ε-caprolactone), PTFE: polytetrafluoroethylene, PMM: poly(methylmethacrylate), PU: polyurethanes, PVC: poly(vinyl chloride), UDMA: diurethane dimethacrylate.

Chromium 51 (^{51}Cr) binds cellular proteins of cultured cells, and its release and accumulation in the medium is an indicator of cell damage.[93] The ^{3}H-thymidine incorporation assay is based on incorporation of the radioactive nucleoside into replicated chromosomes during mitosis. The radioactivity level in the DNA is used as a quantitative indicator of cellular division that occurred during contact with a test agent compared to controls.[94]

These methods have to deal with the problems of handling, storage and half-life of the isotope as well as the need for qualified personnel for radioactivity manipulation.

Apoptosis/necrosis detection. Apoptosis is the mechanism of programmed cell death that occurs to conserve organized cellular structure of tissues; it serves also as a defensive reaction to a cellular aggression induced by diseases or toxic agents.[95] Necrosis is cell death due to disease, injury, or failure of the blood supply and is evaluated by a basal *in vitro* cytotoxicity test.

Apoptotic cells show characteristic biochemical and morphological features including caspase activation, chromatin aggregation, partition of the cytoplasm, and formation of apoptotic bodies containing intact organelles as well as portions of the nucleus.[86] Fluorescent probes can be used to identify cells with high caspase activity in the initial steps of apoptosis that cannot be detected using necrosis assays. During apoptosis, phosphatidylserine molecules (PS) of cellular membranes are translocated to its outer side and are recognized by macrophages, but without membrane disruption in the initial stages. PS exposure on the cell membrane surface can be quantified with Annexin V that binds to PS and stained with a fluorochrome, reflecting apoptotic cells. A variety of dyes such as JC-1, JC-10 or TMRE can be used to measure mitochondrial transmembrane potential changes which take place when mitochondrial permeability transition pores open, inducing the migration of cytochrome C from the mitochondrial membrane to the cytosol and consequently apoptosis. Using suitable nuclear dyes that bind greater amounts of dead cells compared to healthy cells, chromatin condensation of apoptotic cells can be evaluated by microscopy or flow cytometry. DNA ladders can be investigated by electrophoresis; DNA fragmentation, especially the presence of DNA nicks, can be shown by terminal deoxynucleotidyl transferase (TUNEL) assays. For more details on methods for detecting and counting apoptotic cells, readers are invited to read the review by Elmore *et al.*[96]

Other methods. Protease biomarkers,[97] measuring ATP[97] or total protein content are some of commonly used methods in determining cell viability.

A major limitation of *in vitro* cytotoxicity assays for biocompatibility assessment is that a positive result indicating a certain degree of toxicity cannot be extrapolated to anticipate the nature or clinical significance of the observed toxicity. Nevertheless, cytotoxicity assessment is necessary, at least for the validation of the following experiments, and is described in the norm ISO 10993-5.[47] Typically, at least one cytotoxicity assay is performed on each device material.

5.5. Genotoxicity

Genotoxicity tests aim to investigate the genetic damage caused by the biomaterial or its extracts, through the determination of the gene mutations risks implicated either in hereditary defects or in neoplastic lesions. Such assays support the elucidation of the toxicity mechanism and help towards chemical hazard identification.[86]

Genotoxicity evaluation is mandatory for devices having surface contact for greater than 30 days, and greater than 24 hours for implant devices.[12] All the recommended tests within international standards are derived from OECD assays[108] originally designed for chemical hazards. Both European and American standards recommend the use of at least three *in vitro* assays for genotoxicity assessment when applicable covering the three levels of genetic toxicity (DNA damage, mutations, and chromosomal defects).[52] ISO standard 10993-3 recommends methods for mutagenesis evaluation using bacteria (OECD 471) or mammalian cells (OECD 476) in addition to one test for chromosomal damage induction in mammalian cells (OECD 473), with the possibility of using only the first 2 tests if a mouse lymphoma assay (OECD 476) is included.

5.6. Oxidative stress

Oxidative stress refers to a disturbance in the equilibrium between free radical production and antioxidant mechanisms at the level of individual cells or the whole body. Reactive oxygen and nitrogen species (ROS/RNS) are produced as a reaction to modifications of aerobic metabolism, radiation, heme metabolism alterations or hypoxia (Fig. 6). These are the main radicals responsible for oxidative damage including the oxidation of lipids and proteins, DNA injury and apoptosis when produced at very high levels.[109] ROS/RNS participate in the mechanism of vascular reaction in inflammation and some pathological conditions.[110–113]

Measurement of the total amount of ROS could be considered as a go/no go test for further investigations of oxidative stress. ROS can be detected by electron spin resonance capable of detecting unpaired electrons, or by methods based on the transformation of free radicals into stable molecules.[114]

One of the most employed methods is based on the transformation of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) inside cells into DCFH and then into fluorescent DCF by cellular esterases and ROS successively. The fluorescence intensity measured by flow cytometry is compared to adequate controls in order to determine the relative intracellular ROS production.[115] It is important to evaluate the kinetics of ROS production as the increase of their production can be only transitory at short cultures times due to trypsinization step or to a necessary time for cells adaptation to the substrate.[100]

A variety of cellular enzymatic antioxidant scavengers of ROS, such as superoxide dismutases (SODs), glutathione peroxidase (GPx), catalase, and glutathione reductase (GR) (Fig. 6) participate in the antioxidative defence mechanisms of the

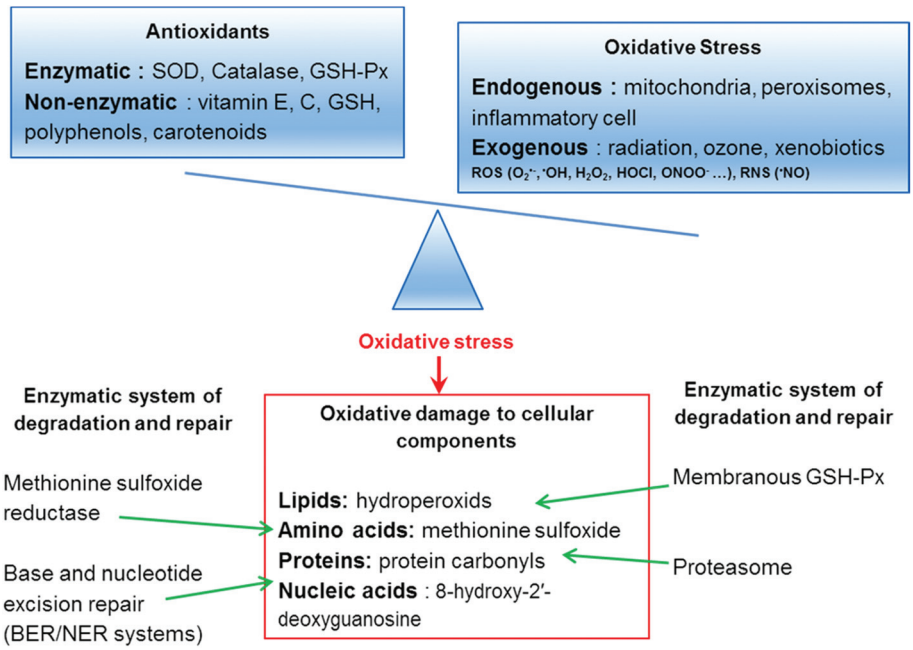

Fig. 6 Oxidative stress balance concept. GSH-Px: glutathione peroxidase, RNS: reactive nitrogen species, ROS: reactive oxygen species, SOD: superoxide dismutase.

cell. Failure of these cellular enzymes to scavenge free radicals may induce apoptosis in a variety of pathological situations including diabetes, cardiovascular diseases, asthmatic bronchitis, ischemic lesions, neurodegenerative disorders and cancer. Measurement of these enzymes levels (quantity, activity, gene expression) could provide a marker of oxidative stress.

GSH determination can be done thanks to the thioreactive fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA) which forms a GSH adduct and is then hydrolysed to the fluorescent 5-chloromethylfluorescein by cellular esterase.[116] Fluorescence is then monitored by flow cytometry. An enzymatic method using Ellman's Reagent (5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)) and glutathione reductase (GR) is able to measure total, reduced and oxidized glutathione: the rate of transformation of DTNB by GSH into a colored product can be followed by optical density measurement and is proportional to total glutathione concentration in the sample. Pre-treatment of the sample with 1-methyl-2-vinylpyridinium triflate, a scavenger of GSH molecules, allows determination of the oxidized glutathione (GSSG) concentration using the same test.[100,117]

SOD activity can be easily assayed in cells and tissues extracts through the induction of superoxide radical that can be either neutralized by SOD or react with nitro blue tetrazolium forming a water-soluble formazan dye. The absorbance intensity in UV is hence inversely proportional to SOD activity.

Other endpoints that can be used in the investigation of gene and protein regulation during oxidative stress include (NFkB, COX-2, Egr-1, JNK, iNOS, c-jun, c-fos, c-myc, *etc.*).[84] Cell membrane and DNA damage, lipid peroxidation or protein inactivation are typical endpoints investigated in this situation.

5.7. Inflammation

Inflammation is a complex adaptive response trigged in a broad range of physiological and pathological mechanisms such as infection and tissue injury.[118] It implicates various signalling pathways, cellular populations and functions, controlled by various mediators.

The inflammatory response to implanted biomaterials referred as "foreign body reaction", is an essential process determining wound healing and implantation success. It starts by an acute inflammation mainly associated with the infiltration of liquid, blood proteins and leukocytes to the perivascular tissues and at the implant site, under the influence of specific signalling molecules produced by inflammatory cells present on the surface of the implanted biomaterial. This process is tightly linked to oxidative stress since inflammatory cells release a high amount of ROS, creating an oxidative environment at the wound site. H_2O_2 gradient in the wound has been shown to act as a chemoattractant for inflammatory cells.[110,112]

Macrophages and lymphocytes are the main cell types found during the chronic phase of the inflammation process which is associated with the development of a vascularised granuloma as part of the wound healing process.

Chronic inflammation state can last under the contentious induction of biomaterial properties and/or its localised movements in contact with surrounding tissues.

Prolonged inflammation around an implant can be detrimental, as activated inflammatory cells can induce serious side effects such as periprosthetic osteolysis or aseptic loosening.

Because of their pivotal role in phagocytosis, the local biological response to implanted devices and their characteristic response to foreign materials with a number of biologically active products secreted, macrophage levels are important for biocompatibility evaluation.[64,119] Investigating their activity and pro-inflammatory cytokines secretion, in contact to biomaterials, is thus a powerful tool to assess biocompatibility. The classical relevant cytokines and factors for the exploration of inflammation are TNF-α, Il-1β, Il-6, MCP-1, MIP-1α, Il-2, VEGF, Il-4 and Il-10[120,121] (Table 3). *In vitro* investigation of cytokines secretion from activated macrophages adherent to different surfaces has been shown to be material and surface dependent while their morphology is related to their activation state.[98] Il-1 and TNF-α are major mediators in the triggering and evolution of the inflammatory process. These mediators can serve as important endpoints to investigate.[119]

According to the chosen cellular model and the biomaterial intended use, it could be useful to explore other chemokines secretion like IFN-γ, Il-13 or NO, expression of NO synthase (NOS) and certain integrin/adhesion molecules (ICAM, VCAM, E-selectin), growth factors secretion (PDGF, FGF, TFG-β, TGF-α/EGF, *etc.*), T-helper lymphocytes Th1/Th2 ratio and to consider biomolecular approaches (gene expression, regulation, *etc.*) for specialised mechanistic comprehension.[1,11,122]

Cytokines are classically quantified by ELISA (Enzyme Linked Immunosorbent Assay), which allows measurement of a single compound at a time. More recent techniques based on the use of flow cytometry together with specific beads functionalized with cytokine-specific antibodies, and associated with distinct fluorophores, are capable of detecting and quantifying various cytokines with an improved sensitivity compared to ELISA assays.[123,124]

Table 3 Examples of inflammation parameters evaluated in polymers biocompatibility assessment

Techniques	Materials	Cells used	Information investigated	References
Morphological means	PU (PEU, PCU)	Human bood monocytes	Cell attachment on the materials surface as an activator marker	Hsu (2004)[70]
Morphological means	Copolymers of PCL and PEG	Human bood monocytes	Number of monocytes *vs.* adherent macrophages	Hsu (2004)[33]
TNFα	Glass PU Glass + chitosan Glass + hyaluronic acid	ThP1	Correlation of macrophage morphology and inflammatory responses	Lee (2013)[98]
TNFα, MCP-1, MIP-1α, Il2, Il6, Il1β, VEGF, Il4, Il10	PE, PU, PVC	Exsudate fluides: neutrophils, lymphocytes, *etc.* from Sprague Dawley male rats after cage implant system	*In vivo* simulation	Schutte (2009)[121]
TNFα, Il6, Il1β, Il4, Il10, GRO/KC, MCP-1, TGFβ in immunoassay; Il2, Il13 in ELISA Macrophages adhesion and fusion	PEU, silicone rubber, PET	Exudate supernatants from Sprague Dawley male rats after cage implant system	*In vivo* simulation, macrophages surface adhesion and fusion	Rodriguez (2009)[126]
- Il-6, Il-8, MCP-1 by ELISA - Leukocyte activation (CD45, L-selectin, Mac-1, CD14) and microparticules formation by flow cytometry	PS, PS grafted with PEG (–OH or $–NH_2$)	HUVEC in coculture with biomaterial-activated whole blood or isolated cells (neutrophils, mononuclear cells, platelets)	Cytokine release into surpernatant from HUVEC Role of each cell's type and microparticules on HUVEC activation phenotypes (ICAM-1, E-selectin, VCAM-1) and cytokine release	Lester (2003)[127]
Il-1β, Il-6, TNFα, MCP-3, MIP-1α, Il-1RA, RANTES, MDC, CCL18	PP, PET, collagen- (COL), PET + COL	Freshly isolated human monocytes (± LPS, IFNγ for stimulation)	Macrophage phenotype in an inflammatory environment: measure of M1-, M2-related proteins and M1/M2 index	Grotenhuis (2014)[128]
IFNγ, Il-4, Il-13 by ELISA Lymphocytes adhesion and subtype identification	PET, PAAm, PAANa	Human monocytes and lympohcytes in coculture	Influence of biomaterial surface chemistries on cytokine production, macrophages and lymphocyte adhesions and lymphocyte subtype selection	Chang (2009)[129]
Il-1β, TNFα, tissue factor (as extrinsic coagulation pathway activation marker) by ELISA	PGS, PLGA, expanded PTFE, Glass	THP-1	Quantification of inflammatory potential of polymers as part of global blood compatibility	Motlagh (2006)[130]

PAAm: polyacrylamide, PAANa: sodium salt of polyacrylic acid, PCU: poly(carbonate)urethane, PDMS: polydimethylsiloxane; PEU: poly(ether) urethane, PEO: poly(ethylene oxide), PTFE: polytetrafluoroethylene, PU: polyurethanes.

Immunohistochemistry is also used to localize and determine the tissue distribution of proteins and inflammatory cells within tissue samples that were in contact with test devices (Table 3).

Murine J774.A1 and RAW264 macrophages are the most used cell lines to investigate inflammatory response to biomaterials. Human THP-1 is a leukemia-based monocytes cell line that can be chemically differentiated into macrophages through the action of phorbol-12-myristate-13-acetate (PMA)[125] and employed for the detection of phagocytosis and/or biomarker secretion in the context of inflammatory responses.

It must be kept in mind that results interpretation have to be done carefully: it is important to distinguish between an excessive increase of cytokine production indicating adverse effects, like those leading to cell death, and the transient increase due to an adaptive phenomenon (immune responses, metabolism modulations, induction of transporters, *etc.*).[86] In order to interpret results with confidence, data concerning the normal levels of explored biological parameters are very important to obtain valid conclusions, and *in vitro* model capability has to be compared to normal *in vivo* cells.

Complement is also a known factor that plays a role in biomaterial-induced inflammation and in local reactions occurring between material and blood or physiological fluids.[131] Indeed, there is a cross link between inflammation and thrombogenic properties in the case of materials in contact with blood (see below).

5.8. Blood compatibility

Hemocompatibility, *i.e.* material compatibility with blood, focuses in priority on blood clotting problems in presence of foreign bodies and on thrombosis-associated risks. It concerns as well elements of the immune system (antibodies, complement system, *etc.*), cells and tissues reaction, including endothelial cells and leucocytes.[132] A blood compatible surface is expected to present no or limited platelet adhesion and activation, to be non-thrombogenic, non-pro-inflammatory and to be pro-healing.

In order to master these difficulties, the international standard ISO 10993-4 describes methods for testing interactions between blood and medical devices for regulatory purpose.[49,62] Those methods are classified into different categories: thrombosis, coagulation, platelets, haematology and immunology.[3] The choice of the tests to perform should be rational and justified based on the context of blood contact (direct or indirect) in addition to the overall contact duration. Regardless of contact time, performing haemolysis, immunology (complement activation) and thrombogenicity testing is recommended. A scientific justification can be made for omitting applicable tests such as complement activation and *in vivo* thrombogenicity in the case of medical devices that contact blood only indirectly.[49]

Although *in vivo* evaluation is inevitable for hemocompatibility evaluation, *in vitro* techniques allow a sufficient number of tests to be performed with low cost and without animal sacrifice. They are powerful tools in an early preclinical stage of device development, useful for the screening of brutal variations of hematological variables, macroscopic thrombosis and the activation of complement system.

Blood samples. Hemocompatibility can be investigated by adequate *in vitro* models using human blood, which is relevant since haemostatic and inflammatory responses are different between human and animal. Freshly collected blood (within 4 hours) is recommended as some of its properties change rapidly once outside circulation. Models for *in vitro* hemocompatibility testing could be static or dynamic. The use of anticoagulants for blood must be carefully thought following the recommendations of ISO 10993-4 based on blood conservation guidelines of the American Association of Blood Banks[133] and European council.[134]

Thrombosis. Thrombogenicity of a medical device is dependent upon surface charge, energy and topography of biomaterials.

The majority of thrombosis evaluation tests are performed *in vivo* (occlusion percentage, flow reduction, thrombus mass, blood pressure drop du to device presence), but some *in vitro* methods permit an indirect evaluation of thrombosis risk that is closely related to protein, leucocytes and platelets adhesion and activation onto biomaterial surface. These methods include SEM microscopy to determine platelet adhesion and aggregation (Table 4) and the use of specific antibodies for the detection of fibrin and/or platelet activation (see below). The detection of microparticle formation (as a consequence of platelets and leukocytes aggregation) by flow cytometry can be an interesting element to understand blood reaction to artificial surfaces.[135]

Coagulation. For coagulation testing, native blood (total, anticoagulated, platelets rich plasma or platelet poor plasma) is exposed to the material under static or dynamic conditions. Both material surface and blood are then investigated. A very simplified method to investigate whole blood coagulation in contact with the biomaterial involves measuring the amount of hemoglobin contained in erythrocytes. Here, measurement is made of hemoglobin not associated with the clot formed at various time points upon coagulation induction of decalcified anticoagulated whole blood.[136]

By using commercial kits based on conventional biochemical techniques, blood coagulation time can be measured. The Prothrombin Time (PT) assay and the Partial Thromboplastin Time (PTT) assay are used to detect variations in coagulation kinetics following the extrinsic and intrinsic pathways respectively.

The amount of thrombin production within the recalcification of anticoagulated plasma can be used as an indicator of contact initiation of the intrinsic coagulation pathway.[137] The measurement of fibrin and fibrinogen degradation product concentrations is an interesting parameter to be evaluated especially in the case of implantable devices where the exaggerated fibrinolysis has to be avoided.

Other tests, suggested by the international standards, include ELISA or radioimmune-detection of specific coagulation factors (fibrinopeptide A, fragment 1–2 of prothrombin

Table 4 Commonly platelet functions assessment for polymers thrombogenicity evaluation

Techniques	Materials	Cells	Information investigated	References
Platelets adhesion and activation	PU (PEU, PCU)	Platelet-rich plasma (PRP)	Number and platelet activation quantified by morphological change	Hsu (2004)[70]
SEM/platelets adhesion; in parallel of a complete hemocompatibility assessment	P4HB films *vs.* PVC	Platelet-rich plasma (PRP)	Platelet morphology on the material surface	Liu (2014)[136]
SEM/platelets adhesion and activation	Copolymers of PCL and PEG	Platelet-rich plasma (PRP)	Number and platelet activation quantified by morphological change	Hsu (2004)[33]
Platelet retention testing with polymer coated bead columns use	4 polyalkyl methacrylates (methyl, ethyl, propyl, butyl)	Platelet in effluent fractions *vs.* whole blood platelet	Relation between fibrinogenconformation adsorbed on artificial surfaces and platelet adhesion	Lindon (1986)[58]
Platelet adhesion/SEM, thrombin generation, recalcified plasma clotting time	Tetraglyme coated surfaces	Platelet-rich plasma (PRP) obtained after centrifugation of ACD anticoagulated whole blood	Number of adherent platelets and morphology by SEM, procoagulant activity of materials, activation of intrinsic coagulation cascade	Cao (2007)[71]
Platelets activation markers (CD61, CD41a, P-selectin) and microparticles (with FITC conjugated Annexin V) by flow cytometry	PS, PS grafted with PEG (–OH or $–NH_2$)	HUVEC in coculture with biomaterial-activated whole blood or isolated cells (neutrophils, mononuclear cells, platelets)	Role of platelets *vs.* mononuclear and neutrophil cells, in endothelial cells proinflammatory phenotype induced by biomaterial (ICAM-1, VCAM-1, E-selectin)	Lester (2003)[127]
Platelet activation *via* detection of β-thrombomglobulin in ELISA	PLLA, PDLLA, P(LLA-*co*-GA), P(DLLA-*co*-GA), P(LLA-*co*-CL)	Platelet-rich plasma (PRP) obtained after centrifugation of citrated whole human blood	Thrombogenicity evaluation of biodegradable polymer materials and influence of surface modification (plasma treatment)	Rudolph (2015)[107]
Platelet adhesion (by quantification with bicinchoninic acid assay measuring the amount of proteins released of films after incubation and washing) and morphology	PU films	Platelet-rich plasma (PRP) obtained after centrifugation of ACD anticoagulated whole blood	Correlation with fibroblasts adhesion and proliferation, proteins adsorption	Faré (2005)[77]
Platelet adhesion (by quantification with LDH amount after cell lysis) Platelet activation *via* detection of soluble P-selectin	PGS, PLGA, expanded PTFE, Glass	Platelet-rich plasma (PRP) obtained after centrifugation of ACD anticoagulated whole blood	Quantification and comparison between polymers as part of global blood compatibility	Motlagh (2006)[130]

ACD: acid citrate dextrose, P4HB: poly-4-hydroxybutyrate, PDMS: polydimethylsiloxane; PCU: poly(carbonate)urethane, PEU: poly(ether)urethane, PEO: poly(ethylene oxide), PGS: poly(glycerol-sebacate), PTFE: polytetrafluoroethylene, PLGA: poly(L-lactide-*co*-glycolide), PU: polyurethanes, PVC: polyvinyl chloride

activation, D-dimer, thrombin/anti-thrombin complex), an increase of these factors indicating an activation of coagulation mechanism. The level of kallikrein and factor XIIf production upon contact with plasma is also a valuable parameter to assess the coagulation system activation.

Platelets. Activated platelets show characteristic changes in their morphology, and release to the blood the content of their α-granules. P-selectin, a soluble adhesion molecule translocated upon activation from the granules to the membrane surface,[138] is released into the plasma[139,140] making of it a molecular biomarker of platelet activation.[141,142] Membrane P-selectin detection indicates the presence of activated platelets on the surface of the device, while the detection of the soluble form takes into account the unbound ones.[130,138] The detection of P-selectin membrane expression can be performed qualitatively through immune florescence or quantitatively by flow cytometry, while ELISA protocols are used for the detection and quantification of the soluble P-selectin. Thrombin can be used as a positive control by inducting P-selectin expression and release.[143]

Morphological modification observed by scanning electronic microscope is the basic method used for the characterization of activated platelets.[130,144] Woolley *et al.* have recently suggested an AFM-based assay for the detection of platelet activation based on morphological changes and aggregation properties.[145] Induced aggregation of platelet from platelet rich plasma under the action of an aggregation agent such as thrombin or collagen, can be used to detect any prolonged aggregation time, caused by the contact with the tested material.

General hematology (hemolysis, leucocytes activation). Leucocyte activation is one of the main mechanisms underlying their migration to an inflammatory site including an implanted material. This activated state can be determined by

the detection of leucocytes surface expressed molecules such as L-selectin that is shed upon activation,[146] which expression can be used as marker of leucocytes activation in response to material contact.[147]

Hemolysis due to the physical or chemical interaction of erythrocytes with the sample is regarded as a significant screening tool. Hemolysis index (HI) is calculated as the percentage of hemoglobin released from erythrocytes in contact with the sample (quantified by spectrophotometry at 540 nm) compared to the total hemoglobin included in the overall erythrocytes used in the assay.[148] Standard ISO 10993-4[49] does not provide a specification of a universal threshold of "hemolysis percentage", hemolysis risk should be compared to the benefice of the device; but according to the ASTM F756, materials can be classified as non-hemolytic when $0 > HI > 2$, slightly hemolytic when $2 > HI > 5$ and hemolytic when $HI > 5$.[149]

Complement activation. The complement system is composed of numerous plasma proteins and its activation induces those associated with immunological cells. Complement activation exploration in human plasma after its exposure to the material is recommended for a variety of blood contacting devices. It is used to determine the extent to which the device under evaluation induces activation of the complement cascade and related inflammatory immune reaction. *In vitro* assays performed with human serum can evaluate the adsorption of complement proteins on material surfaces by the above-mentioned methods in the proteins section. The assay of 50% hemolytic complement activity of serum (CH_{50}) represents the starting point of complement assessment. In this assay, the lysis of modified sheep red blood cells (SRBC) is induced by complement activation of the serum samples pre-incubated with the test biomaterial. Different dilutions of the serum are incubated with the modified SRBC, and the dilution factor corresponding to 50% lysis is calculated, graphed and compared with control serum values. Any decrease in the CH_{50} is considered as a decrease of the complement pathway components.[150] Complement activation could be investigated also by using a C3a generation assay, based on ELISA, immunosorbent or microbeads in flow cytometric methods using highly specific monoclonal antibodies, or SC5b-9 determination for terminal complement complex evaluation.[151] Methods for *in vitro* evaluation for the activation of both classical and alternative pathways using serum are reviewed by Labarre *et al.*[152]

Studies on endothelial cells (EC). Endothelial cells produce a variety of molecules, which participate in the regulation and modulation of the coagulation process.[122] They can be cultured *in vitro* in contact with artificial materials to investigate their adhesion, morphology, proliferation and production of specific biomarkers.

Nitric oxide produced by EC plays an important role in the prevention of platelet aggregation and vascular smooth muscle cell proliferation, which can lead to neointimal hyperplasia. These properties were exploited in the fabrication of polyurethane surfaces covalently linked to diazeniumdiolate, a molecule capable of releasing nitric oxide and improving the hemocompatibility of these artificial surfaces.[153]

Tissue factor (TF) and thrombomodulin (TM) produced by EC and engaged in the initiation of the coagulation cascade can be analysed by ELISA to determine their capacity to convert factor X into factor Xa. In the case of EC contact with test materials, high TM and low TF level are considered as a non-thrombogenic condition.[152]

Other endothelial-related factors that can be analysed in the context of thrombogenicity evaluation include Von Willebrand Factor (vWF), prostacylin (PGI2), tPA/PAI-1, and cell adhesion molecules VCAM-1, ICAM-1, PECAM, E-selectin.[127,154] The aim of these molecular characterization studies is to highlight a pro- or non-thrombogenic phenotype of endothelial cell on certain biomaterials. However, activity studies are more useful in reflecting the complex thrombogenicity process including many molecules at relative amounts. The principal interest of these studies is to make comparisons of expression profiles of these molecules under varying conditions or against various materials, however, this requires comparative investigations between *in vitro* and *vivo* experiments.[155]

5.9. Immunomodulating activity, irritation and sensitization outcomes

The immune system with its various components distributed in all body tissues and organs can be the target of damage induced through contact with medical devices, regardless of the initial contact point.

Immunotoxicity is defined as any adverse effect that impacts the components or functions of the immune system, or other physiological functions due to an immune system dysfunction (sensitization, chronic inflammation, immunosuppression/stimulation, autoimmune disorders). The likelihood of immunogenic substance formation increases with the contact time of the material with the body. However some chemicals will act rapidly and immune responses can appear within less than 24 hours of contact.

Due to the complexity of the immune system and immunological mechanisms, *in vitro* data may not give sufficient information regarding immunotoxicity; nonetheless, this data can help understand toxicity mechanisms when accompanied by *in vivo* data. ISO 10993-20[156] gives guidance on methods for testing immunotoxicity on medical devices and presents a systematic approach for the evaluation of potential adverse immunological effects induced by a medical device. ISO 10993-10[157] recommend the use of validated cellular models such as SkinEthic™ RHE, EpiSkin® and modified EpiDerm SIT®, composed of 3D keratinocytes-based models of epidermis including the stratum corneum, for *in vitro* skin irritation analysis. The end points are the cytotoxicity and the quantification of IL-1α produced by the cellular tissue upon contact with the test device. For *in vitro* assessment of ocular irritation, Bovine Corneal Opacity and Permeability (BCOP) and Isolated Chicken Eye (ICE) are recommended test methods. Internationally recognized assays, such as Cytosensor

Microphysiometer and Fluorescein Leakage assay, are scientifically valid for the investigation of ocular corrosives and potent irritants. The European Centre for the Validation of Alternative Methods (ECVAM) puts considerable effort to develop and validate alternative methods in this field.[158] A variety of *in vitro* methods, originally developed and validated for the evaluation of chemical immunotoxicity, can be suitable for application in the case of medical devices.[159] Chip technology employing chips containing various immune cell types is a promising analytical technique, which can be used for the analysis of specific gene expression variations induced by chemicals.[160,161] For skin sensitization risk evaluation of chemicals, a variety of methods are reported in literature.[162–165] Examples of these methods include measurement of chemical reactivity with glutathione, peptides or proteins with and without metabolic activation[166,167] measurement of chemical activation of keratinocytes[168] or dendritic cells.[169] As mentioned in the WHO guidelines, none of these *in vitro* assays alone can predict the skin sensitization risk. Consequently, a combination of various *in vitro* tests is recommended.

Possibilities for *in vitro* immunogenicity evaluation are limited since models are lacking the immune system complexity and are, therefore, not included in the international standards as part of the biological evaluation plan. Nevertheless, these can be useful as mechanistic studies.

6. Perspective issue

The *in vivo* and *in vitro* assessment of tissue compatibility for biomaterials and medical devices is a wide field that continues to evolve with the development of more sophisticated devices and technologies, as well as improvement in our knowledge of the biological sciences. *In vitro* methods previously described, lead us to keep in mind the following points:

- effect is related to the cell species investigated,

- *in vitro* assessment does not reflect the complete interactions that can be produced with *in vivo* and clinical studies,

- the effect of time is important and only short time evaluation is possible for *in vitro* experiment (commonly 1 to 7 days) due to viability and cells confluence problems; thus, it only reflects a relative immediate effect of biomaterials on cells,

- *in vitro* models even the most sophisticated ones cannot reproduce the complexity of *in vivo* environment such as the immune system and the dynamic blood conditions,

- the use of harmonized methods able to discriminate materials is essential to overcome sensitivity and reproducibility problems.[46]

New challenges for biocompatibility assessment are linked to the actual and future trends in biomaterials and medical devices, which two major examples are:

- Nanoparticules: The high specific surface (surface to volume) ratio associated with nanomaterials imposes special properties that impact their interactions and their *in vivo* fate. Concerns regarding the potential toxicity associated with nanoparticles are numerous. Suitable regulations for their use and evaluation are continuously modified and reassessed, as is the case with the newly approved ISO standard 1099-22 (July 2017) dedicated to the biocompatibility assessment of nanomaterials associated with medical devices. The shape, size, surface charge, dispersion state, matrix composition, surface functionalization, protein corona formation, of nanoparticles have been described to have an impact on cell viability and internalization into cells.[170–172] Those parameters will need to be mastered for biocompatibility assessment with adequate methodology.

- Tissue engineered medical devices and functional materials: A relatively recent approach in which cells or tissue are associated with synthetic materials to regenerate, repair or replace damaged tissues or organs, or induce varied *in vivo* effects.[4,6,173] These new products will offer great advantages for the health and life quality of patients, but at the same time are associated with a higher level of risk compared to conventional medical devices. In this area, immunotoxicity, complex wound healing process and chronic inflammation are the critical factors that need assessment and represent significant challenges in the development of tissue engineered related devices.

In order to optimise the performance and safety of medical implants, and elaborate new devices responding to unmet medical needs, the development of materials associated with well-defined tissue responses is greatly desired. In the past, the biocompatibility of medical devices was considered as the ability to cause no harm to the organism as a result of chemical and biological inertness. But next-generation of medical devices, that control biologic interactions with pharmacological agents, nano-textures, bioactive coatings, *etc*., will require a new biocompatibility concept with new methods for assessment of this positive healing response.[2,173–176] This is an important transformation of biocompatibility paradigm that will need necessary changes in standards and norms, in order to take all these factors into account.

Biological evaluation is an integrative approach that should be based on the complementarities of data obtained thorough chemical characterization, *in vitro* preliminary testing and justified *in vivo* biological investigations, after the evaluation of all previously available data from variable sources. A comprehensive risk analysis of medical devices, through the various steps of biological evaluation as recommended by the ISO 10993-1 standard, is critical. Consequently, both medical device manufacturers and regulatory authorities should implement adequate methods for biological evaluation, including *in vitro* assays, based on justified scientific rationale. This can be only achieved through joint efforts involving all parties implicated in this process, including fabricators, physicians, chemists, material scientists, toxicologists and regulatory bodies.

7. Conclusions

Biomedical applications are increasingly in need of new, lightweight materials with superior mechanical properties and ease

of production. Polymers address many of these requirements with an extraordinary diversity in composition and flexibility in physico-chemical as well as mechanical properties. Any reaction of living cells in contact with foreign materials determines the device performance and safety. Hence, the characterization of specific cell responses through adequate *in vitro* and *in vivo* tests is essential in order to guarantee a clinical controlled performance of the medical device. In this regard, *in vitro* tests represent an essential component of the risk management-based biological evaluation plan, as recommended by ISO 14971 standard.

Current risk management approaches are mandatory at every step of the development plan of the product. The use of validated and standardized test methods, including those recommended in the international standards, enables evaluators to compare results and obtain conclusive data in term of biological safety of test materials. Normative aspects of material safety must be fully appreciated by those working in industry. Early discussions with authorities on biocompatibility test plans in the development stages are critical.

In vitro methods for biocompatibility and biofunctionality evaluation of materials are performed mainly for the assessment of cytotoxicity, hemocompatibility, carcinogenesis and/or mutagenesis, irritation and cell function.

In vitro studies do not replace *in vivo* testing in animals and in humans for the final medical device approval (above the efficacy requirement needed), but are realized as a preliminary evaluations to identify and minimize the number and expense of required *in vivo* investigations. They can be used for screening purposes and to obtain useful quantitative data that help to understand *in vivo* observations (determination of toxic compounds in the sample, toxicity mechanisms) with high sensitivity. A highly toxic material *in vitro* can be replaced in the early development steps with less toxic components. Only complementary data obtained from literature, *in vitro* testing and *in vivo* evaluation can be a reliable prediction of the biological safety of the biomaterials in contact with the host.

Polymeric biomaterials are, more than ever, used in the elaboration of new and sophisticated devices for a variety of health care purposes, including advanced drug delivery technologies. Therefore, global and specific strategies to control, evaluate and improve polymeric-based biomaterial safety and performance have and will continue to evolve. These aspects of medical device biocompatibility evaluations and enhancement have been and remain an area of intensive research.

Conflicts of interest

There are no conflicts to declare.

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