The majority of medical devices available today are designed using relatively inert materials with the purpose of discouraging an aggressive biological response to the devices. It has become clear in the last decade or more that the success of traditional materials in many medical devices is unsatisfactory. Specifically, the recipients of state-of-the-art mechanical heart valves still require life long anticoagulation therapy (Edmunds, 1987), small diameter (<6 mm ID) synthetic vascular prostheses fail due to thrombosis or intimal hyperplasia (Bernex et al, 1992), biosensors work only temporarily because of biofouling, and hip prostheses and dental implants loosen with time because of poor interaction with the surrounding tissue (Cook et al, 1991). However, the economic and legal hurdles to developing new materials are overwhelming for most companies especially in the face of supplier giants such as DuPont pulling out of the field for long term implantation in medical devices such well studied materials as D acron® polyester and Teflon®. These problems have led medical researchers to concentrate their efforts on improving existing materials using surface modification. Ideally, the inherent physical properties of the material are not changed by the modification process.

Two approaches have been taken for optimizing the biologic response of materials using surface modification. The first approach has been to mask the materials with coatings that render them passive or relatively nonadhesive biologically. This approach has been used in applications in which biological deposition needs to be minimal such as vascular catheters, biosensors and other cardiovascular devices. The focus in this approach has been coatings such as fluoropolymers, polyethylene oxide, polyacrylamide, polyvinylpyrrolidone, albumin, pyrolytic carbons and silanes because of their promise for the prevention of an aggressive biological response (Garfinkle et al, 1984; Desai and Hubbell, 1992; Setson et al, 1987; Dunkirk et al, 1991). The success of these passivating coatings has been variable. In fact, recent reanalysis of the platelet response to pyrolytic carbons with state-of-the-art instrumentation has revealed that platelets have an aggressive response to carbons rather than a passive one as was originally thought (Goodman et al, 1995).

The second approach to making existing materials more acceptable biologically has been to modify them with bioactive molecules that allow them to actively participate in the biological interaction. For example, many have studied ways to promote endothelialization of materials used in cardiovascular devices since healthy, functional endothelium provides a physiologic, nonthrombogenic, infection-resistant surface. Modification with cell adhesion
molecules (CAMs) and growth factors (GFs) that promote the attachment and growth of this cell type has been the strategy for encouraging endothelialization of materials (Hubbell et al, 1991; Hubbell, 1993; Twedten et al, 1995; Fischlein and Fasol, 1996; Gosselin et al, 1996). The CAMs, GFs, and other molecules studied for this purpose include arginine-glutamic acid-aspartic acid-valine (REDV)—and arginine-glycine-aspartic acid (RGD)—containing peptides, fibronectin, laminin, heparin, fibrin glue, and fibroblast growth factors. Potentially, any biologically active molecule can be immobilized on material surfaces if the immobilization process is adequately gentle. Whether or not the molecule retains its biological activity needs to be demonstrated on a case-by-case basis. The biologically active molecules that are generating the most interest for surface modification include cell adhesion molecules, antithrombotic/thrombolytic/antiplatelet agents, growth factors, and antimicrobials (Hubesch et al, 1996; Yuan et al, 1995, Greisler et al, 1987, Jansen et al, 1994; Dunkirk et al, 1991).

In contrast to surface modification, traditional materials have also been used as delivery vehicles to deliver drugs to effect the biological response to the device. For example, heparin was shown to minimize thrombus formation associated with mechanical heart valves when delivered from the valve sewing cuff (Schwartz et al, 1973). Basic fibroblast growth factor has also been delivered from expanded polytetrafluoroethylene (ePTFE) vascular prostheses to promote healing (Yamamura et al, 1995). Work has also been done on the delivery of anticalcification agents for the prevention of bioprosthetic tissue valve calcification (Hirsch et al, 1993). For a thorough discussion on the differences between bound versus releasable agents see Hubbell (1993). There are also bulk materials that are considered bioactive such as bioglass, calcium phosphates, and collagens. Some groups have synthesized polymers with bioactive groups such as ProNectin™ which is a genetically engineered silk-like protein polymer incorporating multiple copies of the RGD cell adhesion peptide (Cappello et al, 1990). In addition, with the ongoing interest in heparin as an antithrombotic agent others have reported on new block copolymers that contain heparin (Vulic et al, 1988).

To bring medical devices composed of materials modified with bioactive molecules to market, the bioactive material has to be able to withstand a manufacturing environment which includes assembly, sterilization and long term storage before and after assembly (shelf life). This chapter addresses methods that can be used to characterize materials modified with bioactive molecules to determine their appropriateness for use in medical devices. Specifically, methods for characterization of the nature and uniformity, quantitation, assessment of the biological activity in vitro and in vivo, and determination of manufacturing ruggedness of the surface modified materials are discussed. See Table 1 for an outline of the techniques discussed below. Following this section, the testing that was done to show bioactivity of textiles modified with a cell adhesion peptide for use in cardiovascular devices is discussed. With bulk bioactive materials the testing rationale discussed below should be followed in addition to appropriate physical and mechanical testing to ensure that these properties meet the needs of the device. In addition, as discussed above there is much activity in the development of devices that incorporate controlled release of biologically active molecules in their design. The characterization of this important class of device will not be addressed in this chapter. Methods of surface modification will also not be addressed. A summary on this topic can be found in Hoffman (1987) and Sefton et al (1987). The terms coating and modification are used interchangeably.
Table 8.1. Characterization of bioactive materials

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<td>XPS&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>ISS&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>AES&lt;sup&gt;j&lt;/sup&gt;</td>
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a ATR-IR = attenuated total reflectance-infrared spectroscopy, b SPS = x-ray photoelectron spectroscopy, c ISS = ion scattering spectroscopy, d SIMs = secondary ion mass spectroscopy, e AFM/SFM = atomic scanning force microscopy, f CAMs = cell adhesion molecules, g GF = growth factors, h AT = antithrombotics, i AM = antimicrobials, j AES = auger electron spectroscopy.
Characterization of the Nature and Uniformity of the Modification

Once the bioactive substance of interest has been immobilized or coated on the substrate using covalent coupling (Huebsch et al., 1996), physical adsorption (Twedem et al., 1995), plasma polymerization (Yuan et al., 1995), ion-beam assisted deposition of metals (Sioshansi, 1994), photochemical methods (Dunkirk et al., 1991), and so on, the nature of the modification can be assessed using chemical and surface energetic methods. The information obtained can be used to correlate the surface chemistry with the biologic response to the material and to demonstrate control over the modification process. It is also of interest to assess the uniformity of the modification to ensure that there will be a relatively uniform biological response to the material.

Techniques that are used to chemically characterize surface modified materials include attenuated total reflectance infrared spectroscopy (ATR-IR, Yuan et al., 1995), X-ray photoelectron spectroscopy (XPS, Yuan et al., 1995), secondary ion mass spectroscopy (SIM, static or time-of-flight, for polymer surfaces, ), ion scattering spectroscopy (ISS, Ratner, 1982) and auger electron spectroscopy (AES, for materials other than polymers, Ratner, 1982). See Ratner (1982) for the discussion of additional techniques. Any changes to the morphology or topography of the surface can typically be assessed using scanning electron microscopy (SEM), profilometry (stylus technique, Ratner, 1982) or atomic or scanning force microscopy (AFM, SFM, Huebsch et al., 1996). Many biological molecules can also be labeled with colloidal gold and imaged using SEM (Goodman et al., 1991). Surface energetics can be determined using contact angle analysis (Zisman, 1964). Solid phase immunoassays can be used to determine the uniformity and density of molecules to which antibodies can be raised (Kemeny, 1991; Sheehan, 1990). Autoradiography is a simple way to assess uniformity of radiolabeled materials.

With ATR-infrared spectroscopy the surface modification has to represent at least 5 weight percent of the surface mass in order to get an adequate signal. The absorption of infrared radiation causes characteristic vibrations in materials which are used to construct a spectrum that can be used to determine chemical nature and molecular structure. The infrared beam samples quite deep (1-5 µm) so depending on the coating thickness, the underlying substrate may also be sampled (Harrick, 1979). However, ATR-IR can prove to be a valuable, inexpensive way to characterize the chemical composition of many modifications.

XPS or ESCA (electron spectroscopy for chemical analysis) is a nondestructive technique used to determine the composition of the outermost atomic layers (average depth of penetration is 30 Å) of a material by analyzing the photoelectrons emitted from the x-ray flooded sample. All elements can be detected, with the exception of hydrogen, with a detectability limit of 0.05 atom percent. Chemical depth profiling can also be done with the use of an inert gas sputter ion gun. This technique is especially useful if the coating has different atomic species than the substrate (for example, an amino acid coating on a hydrocarbon can be seen with XPS by the occurrence of a N peak). Static SIMS is a complimentary technique to XPS for qualitatively studying the outermost chemical structure of a solid through the analysis of whole molecular fragments removed by bombarding the sample with a low dose of ions (Ratner, 1982). AES is useful for determining the chemical composition of the top 50 Å of nonpolymeric material surfaces. This technique consists of bombarding samples with electrons and characterizing ejected auger electrons (Ratner, 1982).

Scanning electron microscopic analysis of the substrate before and after modification can be useful for obtaining direct information on the effect of the modification process on the topography of the substrate and/or for imaging the coating. AFM is an extremely sensitive method useful for assessing the three-dimensional structure or morphology of the surface. Under optimal conditions, this technique can resolve detail down to the atomic level (Marchant et al., 1992). AFM images are generated by monitoring the Z-movement of a cantilever with an attached probe tip that is scanned across the sample surface. Typical x-y scan sizes range from
10 by 10 nm to 10 by 10 µm and information such as mean root mean square (rms) roughness and z-range values (lowest to highest points) can be obtained. Some prefer to combine AFM or SFM information with low voltage, high resolution SEM (capable of macromolecular resolution) to ensure that there are no misinterpretations as to the true nature of the modification (Eppell et al., 1995). It is usually best to use a multi-technique approach to fully characterize a surface modified device.

Solid-phase immunoassays are useful for characterizing the uniformity of modifications when antibodies can be raised to the modifying molecule. Substances such as proteins and polysaccharides can stimulate an antibody response if seen as foreign from the host. Obviously it would not be wise to render a material strongly immunogenic. However, peptides and oligosaccharides which are more commonly used in surface modification can be conjugated to the immunogenic substrate keyhole limpet hemocyanin (KLH) to stimulate an antibody response for purposes of tracing the molecule (Harlow and Lane, 1988). Others have biotinylated surfaces and taken advantage of the high affinity avidin-biotin binding complex and the availability of biotinylated antibodies to image surfaces (Blawas and Reichert, 1995). The antibodies can be labelled with fluorescent probes, enzymes for reaction with precipitating colorimetric substrates, or even gold labels for imaging. Coating uniformity can also be evaluated using autoradiography methods which consists of exposing materials with radiolabelled coatings to film at low temperatures (usually at -70°C) for various periods and results in an image of the coating.

Surface energy measurements obtained using contact angle analysis can provide empirical information on how the modification changed the energy and wettability of the surface. This method is sensitive to the very outermost atomic species (top 5 Å of the modification, Olivieri et al., 1992). This technique, when combined with others, can be useful for elucidating the orientation of immobilized molecules (Olivieri and Baier, 1994; Olivieri et al., 1992). Some groups use this technique as a quick assessment of the uniformity or completeness of the modification (Dunkirk et al., 1991).

Quantitation of the Modification

It is necessary to be able to quantitate coatings to determine the optimal amount of coating needed for biological effectiveness and to determine the effect of various challenges on the coating. Radiolabelling is one of the most commonly used methods to quantitate coatings (Huebsch et al., 1996). Nonradioactive quantitation techniques include immunochemical assays, spectroscopy methods, chromatography (if the substance can be reliably stripped from the substrate or if the substrate can be hydrolyzed), fluorescent methods and colorimetric assays. With solid-phase immunoassays, appropriate controls are needed to ensure that background absorption is not an issue. Immunochemical assays are adaptable and can be used for the determination of the amount of coating on substrates by using a soluble substrate (such as orthophenylenediamine dihydrochloride).

Labelling molecules with fluorescent probes is an effective way to trace and quantitate modifications. The general classes of functional groups that can be modified are thiols, amines, aldehydes, ketones and carboxylic acids (Haugland, 1992). Fluorescein isothiocyanate (FITC) is probably the most commonly used fluorescent derivitization reagent. Specific proteins that have been labeled with FITC include actin and epidermal growth factor (Miki and dos Remedios, 1988; Zidovetzki et al., 1981).

ATR-IR spectroscopy can be used to quantitate the coating by using a series of references with known concentrations. It is also useful to look at the density of a coating by IR by monitoring the ratio of a unique band in the coating with one in the substrate (Doillon et al., 1994).
Colorimetric assays have been used for quantitation of many molecules, for example, heparin immobilized on surfaces can be quantitated using the metachromatic dye toluidine blue (Smith et al, 1980). In addition, immobilized glucose oxidase (for detection of glucose) can be quantitated using an o-dianisidine activity assay (Danilich et al, 1992). In short, with many molecules it is possible to exploit their chemistry to tailor assays specific to the bioactive molecule for purposes of quantitation on biomaterials.

Assessment of the Biological Activity In Vitro

The biological activity of the molecule after immobilization can not be assumed, it must be proven. For example, work with heparin immobilization in the past showed that a spacer molecule and end point attachment was necessary to fully preserve the anticoagulant activity of heparin (Ebert and Kim, 1982; Jacobs et al, 1989). The bioassay should assess the biological activities of most importance, for example CAM’s should be tested at a minimum in a cell adhesion assay. It would also be of interest to assess the occurrence of cell spreading and focal attachments with this type of molecule.

Cell adhesion can be quantitated using colorimetric, fluorescent, or radioisotope assays. A typical assay would consist of incubating the cells with either a dye or fluorescent or radioisotope probe, solubilizing the dyed cells with a detergent such as sodium dodecyl sulfate (SDS) and assaying the level of probe released spectrophotometrically as compared to that released from a set of references. For example, one group has developed techniques to covalently label cells with FITC under physiologic conditions. The cells were lysed with detergent and the released fluorochrome was assessed quantitatively with a fluorescence spectrophotometer (Lewinsohn et al, 1988). This same group showed that cell number determination using a fluorometric approach was comparable to that obtained with $^{3}C$ labelled cells. Another group has reported on labelling vascular cells with low concentrations of fluorescent carbocyanine dyes without adversely affecting their proliferative capacity. It is suggested that this technique is applicable to tissue culture assays and perhaps cell detection in vivo (Ragnarson et al, 1992).

Colorimetric probes that have been used for cell quantitation include toluidine blue and crystal violet (Dunkirk et al, 1991). Cell spreading can be imaged with fluorescence, scanning electron, or phase contrast microscopic techniques.

The presence of focal contacts are used to assess the quality of the adhesion of cells to materials modified with cell adhesion molecules. These focal contacts are an extracellular terminus of intracellular actin stress fibers (Burridge et al, 1988). Techniques that can be used to optically image these contacts are interference reflection microscopy (IRM, Gingell and Todd, 1979) and total internal reflection fluorescence microscopy (TIRFM, Axelrod et al, 1982). These techniques are limited to materials that can be preadsorbed onto optical plates. Others have shown the presence of focal contacts using fluorescently labelled antibodies to talin (Huebsch et al, 1995).

Changes in the cytotoxicity of a material as a result of surface modification (specifically to indicate a decrease in cytotoxicity) can be assessed using the industry standard tests extract dilution assay (MEM elution) and agar diffusion assay (Northrup, 1986). In addition, another group reported on the use of fluorescein diacetate and ethidium bromide as fluorescent probes to discriminate between intact and membrane-damaged cells, respectively (Patel et al, 1991). Surface modified materials for cardiovascular applications should also be assessed for changes in blood compatibility and microbe adhesion, especially for CAM and GF modifications (Gosselin et al, 1995).

The anticoagulant properties of antithrombotics should be assessed using appropriate assays such as the inhibition of factor Xa for heparin (Yuan et al, 1995) or more global tests such as activated partial thromboplastin time (APTT), prothrombin time (PT) and so on. Demonstration of nonthrombogenicity of modified materials has been done using platelet assays and
Testing of Biomaterials Modified with Bioactive Molecules: a Case Study

arteriovenous shunt studies (Ito et al, 1992a, Talbot et al, 1989). Many books and chapters have been devoted to the testing of the blood compatibility of materials. Clearly many of the techniques discussed in these references would be appropriate for materials modified with antithrombotics (Leonard et al, 1987; Salzman and Merrill, 1987).

Antimicrobials should be assayed for their ability to kill or inhibit the microbe that is most problematic in the wound the device will be placed (Jansen et al, 1994). Tests for efficacy include adherence experiments (determine if bacteria will adhere to the treated material), zone of inhibition assays and determination of the minimum inhibitory concentration.

Growth factors are potent mitogens of specific cells, so they should be assayed for their ability to stimulate cellular proliferation. Other biological activities of growth factors on specific cells include chemotaxis, cell differentiation, and extracellular matrix production. The activities demonstrated by the soluble growth factor should still be present in an immobilized factor (Burges and Maciag, 1989; Ito et al, 1992b). Osteopontin is an interesting molecule that has been proposed for the enhancement of hard tissue integration of devices due to its cell adhesion and growth factor activities (O’Neal et al, 1992). Platelet-derived growth factor has been shown to influence the orientation of cells on titanium alloy discs for dental implant applications (Lowenberg et al, 1988).

The effect of the surface modification on the function of appropriate cell types (for molecules other than growth factors) can demonstrate the benefits of a modification. For example, for cardiovascular bioactive materials designed to promote endothelialization it may be worthwhile to assess if endothelial cells cultured with the material have the ability to produce prostacyclin which is an indication of their ability to express an anticoagulant phenotype (Jensen et al, 1992). If chemotaxis is an important biological activity of the surface it can be addressed using a modified Boyden chamber (Huebsch et al, 1995) in which the cells are stimulated to move through 8 µm pores towards the chemoattractant. Similarly, modifying half of the surface and assaying for the attraction of cells towards the modified part of the surface can address cell movement activities (Twedten et al, 1988).

In Vitro Challenges

Once the biological activity of the modified material is confirmed under ideal conditions (with an intact, unchallenged coating), these results should be compared to those obtained from modified materials challenged with environments that will be encountered in vivo, for example, to shear conditions of typical vascular grafts or prosthetic heart valves. These flow experiments can be done in saline as a more gentle test and then in plasma to determine if the coating can withstand the detergent effects of plasma. Some have used a rotating disc system to determine the effect of shear on coating integrity. In this system the coating is subjected to many levels of shear in one experiment (from zero at the center of the disc to the maximum at the edge of the disc, the magnitude of which is determined by the rotational velocity and the radius of the disc; Turitto and Leonard, 1972; Horbett et al, 1988).

Kottke-Marchant et al, (1989) reported on an in vitro model for the assessment of blood compatibility of vascular grafts that consists of simultaneously exposing a control silicone rubber circuit and a test silicone circuit that contains the vascular graft to anticoagulated human whole blood. The types of analysis done included cell counts, platelet release measurement, platelet aggregation, APTT, PT and scanning electron microscopy. Others have designed models that simulate left ventricular function to assess the in vitro performance and thrombogenicity of prosthetic heart valves (Swier et al, 1989).

After each of these challenges, the integrity, bioactivity, and amount of bioactive molecule remaining should be assessed using the scheme discussed above. Biological deposition on material
Biomaterials in the Design and Reliability of Medical Devices

Manufacturing Ruggedness

From a manufacturing point of view, of equal importance to showing the ability to modify a material with a biologically active coating is the ability of the modified material to withstand fabrication into a medical device, sterilization and long-term storage (shelf life). Fabrication of the bioactive material into the device typically requires extensive handling with forceps and gloved hands so a coating that easily scratches or rubs off is unacceptable unless it can be shown that the remaining biological activity of the material is adequate.

Ideally, the modified material must also be able to be sterilized after assembly into a device (there is typically more confidence with the FDA in the sterility of a terminally sterilized device, although aseptic assembly is an option). The most common methods of sterilization for synthetics are steam, ethylene oxide (EtO) gas, and ionizing radiation (gamma rays, accelerated electrons). For tissue products the most common method is a mixture of alcohol and aldehydes. Other less commonly used modes of sterilization include plasma gas, peroxygen compounds (hydrogen peroxide, peracetic acid), propylene oxide, and halides in alcohol (Block, 1991).

Bioactive molecules such as antithrombotics and various growth factors typically cannot withstand steam or radiation sterilization and groups have tried less aggressive methods such as EtO or plasma gas. With heparin modified devices, EtO has been shown to decrease the biological activity of the material (data not shown, St. Jude Medical, Inc.). The majority of groups reporting studies in which sterile bioactive devices were tested (e.g., in cell culture systems) addressed sterilization by exposing samples to ultraviolet light (UV), however, UV will not penetrate most substances so it is effective in inactivation of surface bound organisms only. Clearly, this mode of sterilization is not adequate for most, if not all medical devices. Often times it is by trial and error that adequate sterilization methods are found for specific biomolecules.

Finally, once the device is successfully assembled and sterilized using the bioactive material, it must be determined if the device has a reasonable shelf life. Minimum acceptable shelf lives are 4-5 years. Manufacturers do testing in real time and in accelerated conditions of high temperature and high humidity (simulating the device sitting on the loading dock in a tropical environment). After these challenges, it would be advised to assay for the quantity of coating remaining and the biological activity using some of the assays discussed above.

Assessment of the Biological Activity In Vivo

Assessment of the in vivo biological activity of the bioactive material can proceed in two phases; nonfunctional testing in which the material is subjected to the physiological milieu but not put into full function and, if this testing is successful, testing of the material in a final device configuration. An example of nonfunctional testing is using a vascular patch model to assess the healing and thrombotic response to a modified textile for a cardiovascular device application (Twedten et al, 1995). In addition, small animal models such as the rat or rabbit subcutaneous, intramuscular, and intraperitoneal models have been used to quickly and inexpensively assess soft tissue response to bioactive materials (Cholvin, 1986). One group reported on the use of the rat subcutaneous model to assess the infection-resistance of a textile surface modified with an antibiotic. This model involved inoculating a modified textile sample with an appropriate microbe, implanting the sample in the dorsal subcutaneous tissue of a rabbit, and assessing the degree of infection and extent of tissue ingrowth present after one week post-implantation (Ozaki et al, 1993). To quickly determine the calcific potential of surface modified biologic tissue, the weanling rat subdermal model is preferred (Levy et al,
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1991). Hard tissue nonfunctional models include analyzing plugs of the biomaterial placed in long cortical bones such as the humerus or femur (Nillies et al., 1974). These nonfunctional tests are good, economical ways to screen the in vivo response of many bioactive materials. The enhancement of hard tissue wound healing using bioactive molecule coated substrates are typically tested in nonhealing hard tissue defects such as the primate calvarial defects (Ripamonti et al., 1992).

Based on the results of simple in vivo testing then only the most promising bioactive materials are tested in the relatively expensive final device configuration. It is appropriate to choose an animal model that has already been developed if it can be demonstrated using that model that the device fabricated with bioactive materials effects a different biologic response (hopefully improved) than the established control device. For example, the sheep valve replacement model is typically used for the testing of new prosthetic heart valve designs. However, if an improvement in blood compatibility is expected with a surface modification, the sheep has proven to be unsatisfactory because of the difference in the hemostatic systems between sheep and humans. In response to this deficiency, groups are determining the feasibility of the use of a pig valve replacement model since the pig hemostatic system is closer to humans than other nonprimates (Personal communication, Richard Bianco, Director of Cardiovascular Research Laboratory, U. of Minnesota). Vascular grafts are tested in the dog model primarily, but the sheep, pig, and primate model have been used also (Cameron et al., 1993).

Careful thought should be put into the type of evaluation that will be performed on the bioactive device in vivo. For example, Walenga et al. (1992) has performed studies in humans with the Jarvik-7-70 total artificial heart that indicated that an imbalance between the coagulation and fibrinolytic systems (as measured by plasma and cellular activation markers of these systems) was predictive of thrombotic events, in cases where the coagulation and platelet systems were highly activated; and bleeding events, in cases where the fibrinolytic system was highly activated. These same types of relatively noninvasive studies could be done in animal models for the assessment of the blood compatibility of cardiovascular devices if adequate cross reactivity of the antibodies used for the immunochemical studies can be demonstrated or by producing antibodies for the model animal antigens. For a review of other techniques used in hemocompatibility assessment see Sawyer et al. (1986).

Total hip joint replacement and fraction fixation devices have been tested in rats, cats, dogs, sheep and goats (Black, 1992). Dental implants are tested in dogs, sheep, primates, pigs, and rabbits. Good reviews of osteocompatibility and odontocompatibility assessment are found in Tencel et al., 1986 and Natiella, 1986.

As in all experimentation, controls are critical in the testing of these devices. Animal-to-animal variability that confounds comparisons can be accounted for if the device can be configured so that the control and treated portions are part of the same device. For example, a vascular graft could be fabricated from half untreated and half treated fabric. Even so, large numbers of replicates are needed to determine if a statistically significant difference exists between the biological response to the unmodified component and the modified component "interval", "ratio", "success versus failure". The number of replicates needed is dependent upon the type of data collected to assess the biological activity. Smaller sample sizes will be needed if the values of variables are expressed in the «interval» or «ratio» scales rather than reducing data to categories or «success versus failure» measurements. For example, if a device is modified with an antithrombotic agent and the level of thrombus is assessed by percent surface coverage or ranking, larger numbers of replicates will be needed for this analysis as compared to data such as the measure of tensile strength of a bone plate after explantation or the measurement of markers of activation of the hemostatic system after implantation of a cardiovascular device (Machin and Campbell, 1987).
A thorough pathologic analysis of the explants is essential and should include gross and histological workup at a minimum. Electron microscopy may also be worthwhile. Immunohistochemical and histochemical analysis may also be used to identify specific cells or extracellular matrix and cellular activities in response to the bioactive materials (Salthouse, 1986). Schoen discusses special considerations for the pathologic evaluation of cardiovascular devices (Schoen, 1986).

**Immobilization of an RGD-containing Peptide: A Case Study**

Described below is the sequence of testing that was done to determine the suitability of an arginine-glycine-aspartic acid (RGD)-containing peptide for the promotion of healing of cardiovascular medical textiles for applications such as sewing cuffs in mechanical heart valves. The materials modified were polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) fabric. PET and PTFE textiles have shown good success when used in cardiovascular devices, however, the thrombogenic nature of PET and the nonadhesive nature of PTFE led to the exploration of what modification with an RGD-containing cell adhesion molecule would provide these textiles in terms of accelerated, controlled healing.

**Materials**

**Preparation of Substrates**

PET films (Mylar®, DuPont) were cleaned using a series of ultrasonic baths in n-hexanes (J.T. Baker), ethanol (Pharmco), Sparkleen detergent (1%, Fisher), water (reverse osmosis (RO) purified) for 30 sec. each. PET fabrics (Meadox double velour, uncrimped, scoured and heat set; and Meadox® Woven Double Velour Dacron Graft) were used as received. PTFE fabric (Teflon®, Bard, U.S.C.) was used as received.

**Peptide Source**

The peptide investigated was PepTite™ Coating (PepTite, Telios Pharmaceuticals, Inc.). The amino acid sequence of PepTite is Ac-GRGDIPASSKGGGGSRLLLLLR-NH₂ which consists of the cell binding domain, a spacer sequence and an adhesive poly leucine sequence. Its synthesis has been described (Glass et al, 1994).

**Coating and Sterilization of Substrates**

PET and PTFE samples were coated in a dilute solution of the peptide (5-100 µg/ml, stock solution 5 mg/ml in DMSO) in phosphate buffered saline (Dulbecco’s, PBS) for a minimum of one hour with a low level of vacuum (25mmHg). Two, 30 min. rinses were done in PBS followed by two, 30 min. rinses in RO water. The coated substrates were then air dried in a laminar flow hood. Substrates were sterilized using steam for 40 min. at 121°C.

**Cell Adhesion Assay**

MG63 human osteosarcoma cells (ATCC) were used as a “quality control” cell line because of their expression of the appropriate integrins for the specific peptide studied. The cells were cultured in high glucose containing Dulbecco’s modified eagle medium (DMEM, Gibco) with 10% fetal calf serum (Hyclone) 1% L-glutamine, and 100IU/ml penicillin/streptomycin (Gibco) at 37°C in 5% CO₂. Serum free medium consisted of DMEM containing 25 mM HEPES, and 1% ITS culture supplement (Collaborative Biomedical Products). Cells used in this assay were rinsed once with phosphate buffered saline (PBS, 5ml for 75 cm² dish) and trypsinized. The trypsin was inhibited with 2.5 mg/ml soybean trypsin inhibitor (Gibco). Cells
were centrifuged at 1000 rpm and resuspended in serum free medium. Samples were preblocked with serum free medium for a minimum of one hour. Cells (2 × 10⁵) were added to each sample (approx. 1 cm²) and incubated at 37°C for one hour in serum free medium. After incubation, fabric was rinsed with PBS three times to remove loosely adherent cells. Attached cells were fixed in 3% formaldehyde, stained with 0.1% toluidine blue (TB) or crystal violet (CV) for 15 min. to overnight and rinsed with water. Controls were uncoated/unseeded and uncoated/seeded substrates. The number of cells attached were quantitated by solubilizing the stained cells using 1% sodium dodecyl sulfate (SDS) and reading the absorbance of the dye at 630 nm (TB) or 595 nm (CV) wavelength as compared to the dye released from known numbers of stained cells.

**Autoradiographic Assay**

Radiolabelled PepTite (tritiated acetyl group) was used in autoradiographic assays to show coating uniformity and in coating stability assays. Autoradiograms were obtained by exposing substrates coated with radiolabeled peptide to film at -70°C for 24-72 hours.

**ELISA (Enzyme-Linked Immunosorbent Assay)**

This assay was used to monitor coating uniformity and density (qualitatively) without the need for radiolabels. Anti-PepTite™ antibodies were produced in rabbits using keyhole limpet hemocyanin (KLH) coupled to PepTite as the immunogen. PepTite free amines were coupled with sulfo-succinimidyl 4-N-maleimidomethyl cyclohexa N E-1-carboxylate (sulfo-SMCC, Pierce Chemical) yielding a maleamido functionality on the PepTite. A 20 fold excess of sulfo-SMCC was used in this reaction and unreacted material was separated from derivitized PepTite on a reverse phase HPLC column. Separately, KLH was thiolated by the use of 2-iminothiolane (Traut's reagent, Pierce Chemical) and excess thiolating reagent was removed by a desalting column (Sephadex G-25). The derivatized PepTite and thiolated KLH were allowed to react yielding a stable, covalent thioether bond. This material was used as the immunogen with no further purification.

Anti-PepTite™ antibody was purified using the Pierce™ ImmunoPure Plus Immobilized Protein A IgG Purification kit (no. 44679). ELISA assays were derived from Liddell and Cryer (1991) with the following exceptions. The wash buffer consisted of PBS containing 0.05% Tween 20 (Sigma), the blocking solution consisted of PBS containing 3% goat serum (Pierce), 1% ovalbumin (Sigma) and 1% casein (Pierce). The secondary antibody used was goat anti-rabbit IgG horseradish peroxidase conjugate (Pierce). The substrate that was used to form an insoluble colored end-product was DAB (3,3 diaminobenzidine tetrahydrochloride). The intensity of the color produced should correlate to the concentration of the primary antibody and the respective antigen.

**Surface Analysis**

The chemistry of modified PET film and fabric was analyzed using contact angle analysis and X-ray photoelectron spectroscopy (XPS). Contact angle analysis was performed on PET films with a Rame-Hart contact angle goniometer using the method of advancing contact angles. The critical surface tension (γc), dispersive and polar components of the surface free energy (γd and γp, respectively) were calculated by the methods of Zisman and Kaelble (1970 and 1964). XPS was performed on PET films and fabric using a Perkin Elmer Model 5500 XPS spectrometer. Average depth of penetration was 30 Å.
In Vitro Challenges

Stability Assay
Stability assays consisted of incubating tritiated peptide coated fabric in PBS or human plasma (whole blood was citrated 1:10 ratio and centrifuged at 2000 rpm) for 7-9 days at 37°C on a rocker table and determining the amount of coating remaining using a scintillation counter (Beckman).

Sterilization Challenge
This experiment consisted of subjecting coated PET and PTFE fabric to successive steam sterilization cycles to determine how many cycles the coating could withstand and still retain its activity. The coating activity and uniformity was assayed after the challenge using the cell adhesion assay and ELISA, respectively.

Arterial Patch
The arterial patch study was performed as described in Twedén et al (1995). Briefly four uncoated and coated PET and PTFE patches were implanted in the carotid and femoral arteries of dogs for 3 weeks. The animals were heparinized and their vessels containing the patches were perfusion fixed with 2% buffered glutaraldehyde, opened longitudinally, and portions for analysis were retrieved from the middle of the patch and along a longitudinal plane. Specimens were prepared for scanning electron microscopic and histological analysis using standard techniques. The thickness of neointima formed on the patches was quantitated with morphometric techniques.

Valve Replacement Model
Sewing cuffs were assembled using coated fabric under clean room conditions. The bioactivity was assessed using the cell adhesion assay and the uniformity was assessed using autoradiography (data not shown) after cuff assembly to ensure that an excessive amount of coating was not removed as a result of the assembly. St. Jude Medical® mechanical heart valves with either uncoated or PepTite coated polyester sewing cuffs were implanted in the mitral position in 16 juvenile sheep as described in Twedén et al (1995). No anticoagulants were given postoperatively. Between two to four weeks post implantation the valves were explanted after the animals were systemically anticoagulated with heparin. Explanted valves were fixed in Karnovsky’s fixative and the sewing cuffs were analyzed grossly and histologically using semi-quantitative scales. Exposed Dacron, thrombus, thin pannus and pannus overgrowth were scored grossly from 0 to 5 in 15% or 25% intervals (0 poor response and 5 good response). Thin pannus is characterized by a smooth, white—appearing neointima under which the fabric pattern can still be seen. Pannus overgrowth is defined as tissue that begins to encroach on the valve orifice. Two samples for histological analysis were retrieved 180° apart (90° from the ear mechanism) and prepared using standard techniques. Histologic features graded were maturity and thickness of pannus (scale of 1-4 and in µm, respectively), presence of endothelial cells (+ or -), thrombus, deep organization, inflammation and calcification (scales of 0-4). The higher the score the poorer the response for the histological variables.

Results

In Vitro Assays
PepTite was found to consistently promote both cell attachment and spreading in serum-free medium to both PET and PTFE. In contrast, very few cells were found to attach to the uncoated
Fig. 8.1. a) Cell attachment to uncoated PET fabric.

Fig. 8.1. b) Cell attachment to PepTite coated PET fabric using cell adhesion assay described above.

samples and those that did had a round morphology (Figs. 8.1 and 8.2). Specifically, PepTite was shown to promote a 5 fold increase in cell attachment on the PET substrate as compared to its uncoated control and a 3.5 fold increase on the PTFE substrate as compared to its uncoated control (Fig. 8.3).

Both autoradiography and ELISA were used to assess the uniformity of the PepTite coating. Autoradiograms are shown in Figure 4. Close replications of the PET and PTFE fabric patterns were generated on the film indicating coating uniformity. Longer film exposures are needed for PTFE samples since it quenches radiation. Samples subjected to ELISA are also
Fig. 8.2. a) Cell attachment to uncoated PTFE fabric

Fig. 8.2. b) Cell attachment to PepTite coated PTFE fabric using cell adhesion assay described above.
Fig. 8.3. Graph showing quantitation of cell attachment using a colorimetric method.

Fig. 8.4. Top two rows: Autoradiogram of PepTite coated PET fabric (left) and PTFE fabric (right, top row is 24 hr exposure to film, second row is 1 week exposure to film), bottom row: ELISA of uncoated (left two) and coated PET fabric (right two).
Fig. 8.5. Coating stability in plasma

Table 8.2. Contact angle analysis of PepTite modified Mylar® PET

<table>
<thead>
<tr>
<th>Sample (n)</th>
<th>Conc. PepTite (µg/ml)</th>
<th>(Y_c) (dynes/cm) (SD)</th>
<th>(Y_p) (dynes/cm) (SD)</th>
<th>(Y_d) (dynes/cm) (SD)</th>
<th>Water Contact (?)(°)(SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET (n = 3)</td>
<td>0</td>
<td>40.0 (3.4)</td>
<td>6.9 (1.2)</td>
<td>31.6 (3.0)</td>
<td>85 (10)</td>
</tr>
<tr>
<td>PET (n = 2)</td>
<td>5</td>
<td>35.5 (3.8)</td>
<td>19.1 (0.4)</td>
<td>23.6 (1.0)</td>
<td>62 (0)</td>
</tr>
<tr>
<td>PET (n = 2)</td>
<td>50</td>
<td>33.9 (0.5)</td>
<td>16.1 (0.8)</td>
<td>26.3 (1.0)</td>
<td>62 (0)</td>
</tr>
</tbody>
</table>

shown in Figure 8.4. The even brown color seen on PepTite coated PET samples indicates a relatively uniform coating. Results were similar on PTFE samples (data not shown).

The stability data for PepTite coated PET in plasma are shown in Figure 8.5. The coating was seen to be quite stable in both saline and plasma, with only an initial loss followed by a plateau. The amount of peptide remaining was found to promote cell attachment using the cell adhesion assay (data not shown). The amount of coating before the challenge was 157 µg/mg PET fabric and after 7 days of challenge was 119 µg/mg. The sterilization challenge showed that PepTite coated PET fabric could withstand five, 40 minute steam sterilization cycles (121°C, 15 psi) with no loss in coating uniformity or biological activity (evaluated with ELISA and cell adhesion assay, respectively, data not shown).

Contact angle analysis showed that PepTite modification of PET resulted in an increase in hydrophilicity, a decrease in critical surface tension (\(\gamma_c\)), and an increase in polarity (\(\gamma_p\)). Data are shown in Table 8.2. PepTite modified germanium was found to have similar surface ener-
Testing of Biomaterials Modified with Bioactive Molecules: a Case Study

The nitrogen (N)-containing amide bond of the peptide coating was shown by XPS. Also the higher the initial coating concentration, the higher the N signal seen by XPS (Table 8.3).

### In Vivo Data

Scanning electron micrographs showing the typical healing response to the luminal side of uncoated and PepTite coated PET and PTFE patches are shown in Figures 8.6 and 8.7. On the uncoated patches, the response is characterized by a platelet/fibrin mesh, rounded leukocytes and spreading cells which appear to be macrophages. In contrast, the PepTite coated patches were characterized by a complete lining of the luminal side by cells that had surface morphology characteristic of endothelial cells (Figs. 8.6B and 8.7B). The morphological characterization showed that 75% (3 out of 4) of the PepTite coated patches were covered with endothelial-like cells whereas only 25% (1 out of 4) of the uncoated patches had a similar lining. Openings of vasa vasorum which are lined with elongated endothelial-like cells were seen on the healed surfaces.

Typical histological sections through the explanted patches are seen in Figure 8.8. These sections were taken longitudinally, so that the characteristic hills and valleys of the crimped fabric pattern can be seen. The measurements for the neointimal thickness were taken along the patch at the "hills" of the graft away from the anastomosis. The neointimal thickness measurements showed that the pannus thickness on PepTite coated PET patches was essentially half of that seen on uncoated patches (mean of 120 vs 66 µm). No difference in neointimal thickness was seen between uncoated and PepTite coated PTFE patches. The extent of foreign body giant cells seen associated with the PET fibers was reduced noticeably in the PepTite coated PET patches as compared to the uncoated patches (Figs. 8.8A and 8.8B).

Representative gross macrographs of valves with uncoated and PepTite coated polyester cuffs explanted at approximately 2.4 weeks are shown in Figure 8.9. The uncoated cuff healing was characterized by thrombus build-up and exposed Dacron fabric. In contrast, the coated

### Table 8.3. Atomic concentrations (%) of PepTite modified Dacron® and Mylar® (standard deviation)

<table>
<thead>
<tr>
<th>Sample</th>
<th>C</th>
<th>O</th>
<th>Si</th>
<th>N</th>
<th>F</th>
<th>Na</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dacron (n = 3)</td>
<td>72.7</td>
<td>27.1</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(1.3) (1.2) (0.09)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dacron 5 µg/ml</td>
<td>70.8</td>
<td>25.9</td>
<td>0.2</td>
<td>3.2</td>
<td>0.1*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PepTite (n = 2)</td>
<td>(0.85)</td>
<td>(0.9)</td>
<td>(0)</td>
<td>(0.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mylar (n = 2)</td>
<td>87.3</td>
<td>12.3</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(14.9) (14.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mylar 5 µg/ml</td>
<td>72.7</td>
<td>23.4</td>
<td>1.0</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PepTite (n = 2)</td>
<td>(2.4)</td>
<td>(2.7)</td>
<td>(0.4)</td>
<td>(0.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mylar 50 µg/ml</td>
<td>74.3</td>
<td>21.3</td>
<td>0.5</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PepTite (n = 3)</td>
<td>(7.3)</td>
<td>(6.9)</td>
<td>(0.1)</td>
<td>(0.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = detected in one sample only.
ND = not determined.
Figs. 8.6A,B. Scanning electron micrograph of luminal side of explanted A) uncoated PET and B) coated PET vascular patch.
Fig. 8.7. Scanning electron micrograph of luminal side of explanted a) uncoated PTFE and b) coated PTFE vascular patch.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Gross thin pannus</th>
<th>Histologic pannus thick (μm)</th>
<th>Histologic pannus maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>411&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PepTite coated</td>
<td>4.1</td>
<td>301</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.001, <sup>b</sup> p = 0.04, <sup>c</sup> p = 0.04
Figs. 8.8. A) Histological section of uncoated PET vascular patch, B) Histological section of coated PET vascular patch.

Cuff healing was characterized by a greater amount of white, smooth pannus that appeared endothelialized at this time period. Overall, the significant differences seen in the gross and histological data were the following: a greater extent of thin pannus formation on the inflow side of the coated cuffs compared to that on the uncoated cuffs (mean of 4.1 and 2.1, respectively, \( p < 0.001 \)), a 27% thicker pannus on the uncoated cuffs (411 mm) compared to that seen on the coated cuffs (301 mm, \( p=0.04 \)), and finally, more advanced pannus maturity on the coated cuffs at 3 weeks (1.9) than that seen on the uncoated cuffs (2.6, \( p=0.04 \)). These findings indicated an accelerated and more controlled healing to the PepTite coated PET sewing cuffs compared to that seen with the uncoated cuffs (Table 8.4).
Discussion

The methods for the testing of materials modified with bioactive molecules for medical devices were outlined. The results using the outlined testing strategy, on fabrics modified with an RGD-containing peptide for use in cardiovascular devices were discussed. First it was shown that the coated fabric had acceptable bioactivity using the cell line MG63 osteosarcoma. PepTite has also been shown to promote the attachment and spreading of an endothelial cell line which is a cell type more relevant to the tissue of interest (data not shown). The coating was shown to
be uniform in nature using the autoradiogram assay and ELISA which was shown to be important for uniform cell response (data not shown). The coating amount was quantitated using radiolabelled peptide (this technique could be used to determine the minimum amount of coating needed for adequate biological activity). The chemical nature and surface energetics of the coating were analyzed using XPS (the coating could be seen with the presence of a N peak) and contact angle analysis (the coating was more hydrophilic and polar in nature than the substrate), respectively. It was found that the coating could not be detected using electron microscopic or ATR-IR analysis since only low concentrations were needed for acceptable biological activity on the materials (data not shown). The coating was also shown to be able to withstand biologic (buffer and plasma shaker bath storage) and manufacturing (successive sterilization cycles and fabrication into cuff prototypes) challenges with little decrease in coating activity. Finally, the "ultimate" challenge of in vivo biological activity using a simple vascular patch model and a valve replacement model showed that the coating accelerated wound healing corroborating the in vitro cell adhesion data. The biological significance of such a coating in a mechanical heart valve application is discussed elsewhere (Twedet al, 1995).

In conclusion, a full range of testing is necessary to determine on a case-by-case basis if materials modified with bioactive molecules can be used successfully in medical devices.

Acknowledgements

Thanks are extended to Sheila Kelly and Dan Langanki (St. Jude Medical) for performing cell adhesion assays, ELISA, and sterilization challenges. Thanks are also extended to former and present personnel from Telios that performed radioisotope studies and were involved in the in vitro work direction, specifically Jonathan Blevitt, James Glass, Kenneth Dickerson, William Craig, Ph.D., and Michael Pierschbacher, Ph.D.. Hiroaki Harasaki, M.D., Ph.D. from the Cleveland Clinic Foundation performed the vascular patch study and Michael Jones, M.D. from the NIH performed the valve study.

References


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