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Vascular prostheses with controlled release of antibiotics Part 2. *In vitro* biological evaluation of vascular prostheses treated by cyclodextrins

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Abstract

Viability tests by the colony forming method show no toxicity for all CDs (β-CD, γ-CD, HPβ-CD and HPγ-CD) and their associated polymer. A survival rate of 100% is observed for all CDs at high concentration 400 ppm. Proliferation tests revealed a low proliferation of L132 cells on grafted vascular prostheses and untreated prostheses and good proliferation on Melinex® (film form of PET). A proliferation of 17% is observed after 3 days of incubation and decrease at 4% after 6 days on prostheses. Melinex® exhibits a proliferation rate as the controls. Vitality tests confirm proliferation tests and show a good vitality of cells even for low cell amounts. From these experiments it becomes obvious that the decreasing proliferation rate is not a cytotoxic effect but is due to the chemical and/or physical surface characteristics. A similar result is obtained for cell adhesion kinetics between grafted vascular prostheses and control. After 2 h adhesion, a lower adhesion is observed on untreated prostheses. Theses results were confirmed by immunochemistry and morphology tests. This cell adhesion inhibiting effect of the PET prostheses contributes to a better ''survival'' of vascular prostheses without secondary obstruction or stenosis.

Keywords: PET vascular prosthesis; Cyclodextrins; Grafting; Biocompatibility; Toxicity; Adhesion

1. Introduction

Vascular prostheses made of woven PET (Dacron®) are used since about 40 years in the aim to replace or bypass damaged arteries in patients with peripheral arterial disease or aneurysms (Haulon et al., 2003). Unfortunately, some complications may arise after implantation such as infections mostly associated with high mortality rates (O'Brien and Collin, 1992). In order to reduce this risk, we developed a new vascular prosthesis functionalised with cyclodextrins. Cyclodextrins are cage molecules, which can encapsulate in a short time many molecules and liberate them in a prolonged time due to hydrophobic interactions between the internal part of CD and the active molecule. In order to reduce the number of infections, we grafted CDs on vascular prostheses and loaded them with antibiotics in order to have a controlled release in a curative time (Martel et al., 2006). Previous studies showed the possibility of cyclodextrin grafting (β-CD, γ-CD, HPβ-CD and HPγ-CD) on woven polymer (PET, polypropylene, cotton, etc.) (Martel et al., 2005). Grafted tight PET prostheses were obtained with cyclodextrins and a thin coat of collagen. Mechanical tests revealed an excellent resistance of grafted prostheses after longitudinal and circumference traction and they were totally watertight and blood tight (Blanchemain et al., 2005). To implant this new prosthesis in human duty, the prostheses have to be non-toxic. All tests with cyclodextrins reveal non-toxicity for high concentrations. For β-CD, tests were performed on dog and reveal none oral toxicity of this cyclodextrin until 1831 mg/kg/day for male dog (Bellringer et al., 1995). For γ-CD, no oral toxicity until 7700 mg/kg/day and no i.v. toxicity until 200 mg/kg/day for male dog (Til and Bär, 1998; Donaubauer et al., 1998). Similar results have been reported for HPβ-CD and γ-CD (Brewster et al., 1990).

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2. Materials and methods

Polyester (polyethyleneterephtalate, PET, Dacron®) prostheses were manufactured by Laboratoires Perouse (Ivry-Le-Temple, France). They are commercialised as Polythese® (knitted fibres, 2 yarn of 100 dTex, diameter 24 mm, crimp, surfacic weight = 133 g/m^2, wet pick up = 90%).

Melinex® was supplied by Dupont Teijin Films™, it is free of any additives. The PET film had a thickness of 250 μm and was biaxial oriented. Before sterilisation, the surfaces of PET were ultrasonically cleaned with ethanol for 20 min.

γ-CDs and hydoxylpropyl-γ-CDs (HPγ-CDs) were provided by Wacker Specialties GmbH (Cavamax®, Burhausen, Germany), β-CD and hydroxylpropyl-β-CD (HPβ-CD) by Roquette (Kleptose®, Lestrem, France). Citric acid (CTR) and sodium dihydrogen hypophosphite were Aldrich chemicals (Milwaukee, WI, USA).

Polymers of cyclodextrins were prepared by solubilization of the catalyst, cyclodextrin and acid in standard condition (Martel et al., 2005).

For biological tests, disks of 15 mm in diameter were cut from each untreated and grafted PET prostheses and Melinex®. PET prostheses were grafted with a rate of 10 wt.% and one thin coat of collagen. Negative and positive control is, respectively, Thermanox® and Nickel.

For viability test, PET is used in a powder form obtained by mechanical grinding under liquid nitrogen (deep freezing method). The particle size <20 μm was determined by granulometry. CDs and polyCDs were used in soluble powder form.

2.1. In vitro experiment

In vitro cell tests were performed with human embryonic epithelial cells L132 (ATCC CCL5), following the International and European standards (ISO 10993-5/EN 30993-5). L132 cells were cultured in minimum essential medium (MEM 0011) with glutamax (Gibco BRL), supplemented with 5% foetal calf serum (FCS) (Eurobio) (Hornez et al., 2002). All media contained streptomycin (0.1 g l^{-1}) and penicillin (100 IU ml^{-1}). All *in vitro* cell incubations were performed at 37 °C in 5% CO_2 atmosphere and 100% relative humidity in a Binder CO_2 incubator (CB 150/APT.line) with high stability of all technical parameters.

2.2. Viability test

The viability tests consisted of the establishment of the relative plating efficiency (RPE) and subsequently, the 50% lethal concentration LC50 (or RPE 50) by using the colony-forming method with L132 cells (Frazier and Andrews, 1979). The L132 cells were selected for their good reproducibility and their cloning efficiency of about 37% (Puck and Marcus, 1955). Cells were continuously exposed in MEM medium supplemented with 10% foetal calf serum (FCS) to increasing concentrations of PET powder (0, 200 and 400 μg ml^{-1}), and of CDs and polyCDs (0, 25, 50, 100, 200, 400, 1000, 2000, 4000 ppm) without renewal of the growth medium during the experiments. After 9 days, the medium was removed and the colonies were coloured with crystal violet. The clones were counted with a binocular microscope. At least eight repeated experiments were performed in triplicates for each concentration. Results are expressed as the mean values ± S.D. with respect to the control (100%), and are compared with nickel as positive control.

2.3. Cell proliferation

The growth period for cell proliferation tests was 3 and 6 days without renewal of the medium (Hornez et al., 2002). PET disks were placed in the bottom of 24-well plates (Nunc) after gamma sterilization. Viton® rings were added to maintain the samples on the bottom of the wells and subsequently to avoid cells growing under the test samples. Then 10^4 growing cells were gently seeded in each well. Wells filled only with cell suspension served as control. After detachment of cells in a trypsine-EDTA solution, their number was determined using a cell counter Z1 (Coulter Electronics). Three samples of each group are used for one test. Results are expressed as the mean percentage of four separate triplicate assays for each PET type.

2.4. Cell vitality

Cell vitality test was assessed with the non-toxic Alamar Blue dye simultaneously to the proliferation test (O'Brien et al., 2000). Three and 6 days after the cell seeding, and just before cell counting, the culture medium was removed from each well. A 500 μl of the diluted fluorescent dye (Interchim Ref. UP669413) was deposited in each well. After 3 h in the incubator, the solutions were transferred into 96-well plates (Nunc), and the absorption was measured by fluorescence (Twinkle LB970™—Bertold) at 560 nm. The cell vitality rate was calculated as the absorption of living cells on PET samples divided by the absorption of control cells. Data were expressed as the mean percentage ± S.D. with respect to the control culture (100%).

2.5. Cell adhesion

For cell adhesion test, 4×10^4 growing cells were gently seeded in each well. Two hours later, the wells were emptied, and 300 μl of a dye (*p*-NPP: *para*-nitrophenyl phosphate, Sigma®) were deposited (Trochon et al., 1997). After 3 h incubation, 150 μl of 1N NaOH were added in each well to stop the reaction. A 200 μl were then removed from the wells and transferred in a 96-well plate (Nunc). The absorption was read by fluorescence at 405 nm (Apollo LB911™—Bertold). Four different triplicate assays were run for each test. The results of cell adhesion are compared to the control (wells without cells).

2.6. Fluorescence microscopy

Two different proteins have been visualised by fluorescence microscopy: cytoskeletal actin and vinculin contained in focal adhesion contacts. After 3 days, the cells were stained for 20 min in 2% *para*-formaldehyde at room temperature, permeabilized in a PBS-Triton X-100 buffer (Sigma), and then blocked with 1% bovine serum albumin (Sigma) in PBS (Sigma). Cytoskeletal organisation: Actin filaments were directly labelled with 1.2 μg ml^{-1} FITC-phalloidin (Sigma). Focal contact formation: Cells were immune stained with 300 μl of a 1:50 diluted mouse anti-chicken monoclonal antibody against vinculin (Sigma) followed by FITC-rabbit-anti-mouse antibody (Sigma) (Linez-Bataillon et al., 2002).

After washes in buffer, the specimens were embedded in PBS-glycerol–DABCO (1:1) (Sigma) mounting medium and examined in a LEICA TCS NT LASER scanning confocal microscope. For these observations, cell nuclei were previously labelled with propidium iodide (Sigma).

2.7. Scanning electron microscopy

Cells were stained with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffered, pH 7. After two washes in the same buffer, they were post-stained with 1% OsO_4 in saturated $HgCl_2$, then dehydrated in graded ethanol, critical point died, and finally sputtered with gold/palladium. Surface morphology of untreated and grafted PET prostheses and cell morphology were observed with a SEM (JEOL J-SM-5300), using an accelerating voltage of 30 kV and a current of 100 μA.

3. Results

3.1. Viability tests

The first biological test was carried out to determine the non-toxicity of all samples we used, i.e. PET, CDs and polyCDs. The powder of PET showed an excellent biological behaviour with a survival rate of 92% at high concentration (400 ppm) (Table 1).

Soluble powders of β-CD, HPβ-CD, γ-CDs and HPγ-CDs exhibited an identical, i.e. excellent biological behaviour up to high concentrations (400 ppm) with survival rates of 95–107%. At a extreme high concentration (4000 ppm), only β-CD