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Formulation and *in vitro* characterization of a composite biodegradable scaffold as antibiotic delivery system and regenerative device for bone

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ABSTRACT

A biodegradable composite scaffold, useful as tridimensional bone regenerative scaffold (3D) and antibiotic delivery system (DDs), is here formulated and characterized. The device, called as 3D-DDS, is an injectable or moldable scaffold based on chitosan chloride crosslinked with glycerol 2-phosphate disodium salt and laden with bovine bone substitute (BBs) granules and gentamicin loaded polylactide-co-glycolide-co-poly ethilenglycol (PLGA-PEG) microparticles. 3D-DDS characterization results show that microparticles are homogeneously distributed in the composite matrix, and do not interfere with scaffold stability. The lyophilized 3D-DDS has high rehydration ability and is able to restrain physically bovine bone granules and microparticles up to 45 days in *in vitro* simulated conditions. Bactericidal action is high in the first 4 h and it accounts for superimposable effect of gentamicin release and bacteriostatic chitosan properties; sustained gentamicin bactericidal effect is evident up to 14 days.

1. Introduction

Surgical site infections in orthopedic surgery and bone trauma account for about 15% of all nosocomial infections [1]. In particular, osteomyelitis is a chronic recurrent infection that can rise after open fractures and implant-related infections or following treatment of fractures and prosthetic joint replacements. Osteomyelitis can be treated by medical or surgical therapy, and in the worst-case amputation of the infected part is needed with consequent reconstruction of the resected tissue. The problem is significant in the case of periprosthetic implant infections, known as septic failures, because of prolonged hospitalization, high risk of relapses with poor healing outcomes, and high costs involved in the treatments, which amount to $70,000/per episode in USA [2].

The treatment usually undergoes several stages: drainage of the site, debridement of the necrotic tissue, bone dead space management, soft tissue coverage, and restoration of blood supply. The most important and critical step is the management of dead space, namely the void space between soft tissue and bone defect generated from surgical resection. An appropriate management of the dead space reduces the risks of persistent infection at the originally infected site and of distortion of surrounding tissues maintaining the physical integrity of site. Generally, the dead space management is performed by insertion of autologous vascularized tissue, soft tissue transplant, antibiotic impregnated bandage and bone grafting. Currently, antibiotic loaded beads represent the gold standards for the dead space management; they are based on inert material, polymethylmethacrylate (PMMA), and loaded with a broad spectrum antibacterial agent such as gentamicin. They are easily inserted in the required body cavity; nevertheless, they need to be removed when exhausted by a second surgery with associated risk of further infection, painful and high cost [3].

In spite of the innovative treatments, the literature highlights that chronic osteomyelitis is still a challenging condition to treat [4]. Recurrence of infection is well known, and successful treatment

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needs a multidisciplinary team approach.

Since one therapeutic approach is the bone grafting with implantation of natural bone substitute, the goal of the present work is to design a bone grafting combined with a drug delivery system (3D-DDs). The 3D-DDs could be injectable or moldable and it is composed by natural biocompatible biodegradable polymer combined with decellularized bone matrix (Bovine Bone substitute-BBs) and with gentamicin loaded biodegradable microparticles.

The advantages of 3D-DDS are multiple, it is ready to be used, injectable through a cannula or inserted through minimally invasive surgical procedure, easy to be placed into irregular body cavities and to fit perfectly irregular site maintaining its physical integrity.

The moldable 3D scaffold is designed for invasive surgical procedures; it can be cut, hydrated and extemporaneously remodeled by surgeon in order to fit the bone defect. It is obtained by lyophilization of injectable composite scaffold and the high porosity and the spongy like structure typical of lyophilized scaffolds allow the rapid hydration of scaffold in blood or physiological fluids unchanging its physical structure.

The 3D-DDs represents a new generation of surgical scaffolds obtained by combination of bone grafting and drug delivery system, they can be useful in orthopedic medical practice because they can provide the prevention or the complete treatment of infection improving, simultaneously, the tissue regeneration reducing the healing process. The injectable and mouldable 3D-DDSs present several advantages: they are biodegradable, biomimetic and they promote local and extended gentamicin delivery to the bone. Moreover, they could be loaded with antibiotic combinations reducing the risk of bacterial resistance, and due to their *in situ* gelification, they can create intimate contact with the surrounding tissue promoting the diffusion of stem cells and avoid the failure of surgical procedure due to the cavity distortion. Furthermore, the amount of antibiotic loaded into the system can be adjusted in order to achieve its desired time specific release profile. Eventually, the local delivery of the antibiotic at the site of action can dramatically reduce the antibiotic side effects arising from a systemic administration.

The potential limitation and/or drawback of the proposed 3D-DDs could be associated with potential accumulation phenomena of antibiotic due to a slow residual release of antibiotic, which can evoke bacterial resistance. This critical aspect has been considered and evaluated in the present work.

Gentamicin (Gent) has been chosen since it is a wide bacterial spectrum aminoglycoside antibiotic extensively used for treating many types of infections including osteomyelitis. It has plasma half-life of 2 h in patients with normal renal function, while its half-life in the renal cortex is 100 h. Systemic administration of gentamicin can rise diverse problems and side effects. The antibiotic has low bioavailability after oral administration and poor cellular penetration; in addition, the internalized gentamicin molecules are accumulated into the lysosomal compartment with reduction of its activity. Since it is excreted by glomerular filtration, repetitive doses of gentamicin can result in renal accumulation and nephrotoxicity. Due to these characteristics and criticalities, gentamicin is a good candidate for local administration, in order to reduce side effects caused by systemic administration whilst improving the therapeutic ones.

Biodegradable biocompatible polymers have been widely investigated as carriers for gentamicin because of their advantageous properties [5–8]. polylactide-co-glycolide-co-polyethylenglycol (PLGA-PEG) has been selected as biodegradable, biocompatible constituent of the microparticle matrix, the presence of polyethylene-glycol (PEG) gives the hydrophilic-lipophilic molecular balance, suitable to improve gentamicin loading, and fosters microparticles loading into the chitosan hydrogel. The preformulative and formulative phase, involving the optimization of gentamicin loaded microparticles preparation method, has been carried out in a previous work throughout design of experiments (DOE) together with the characterization of gentamicin loaded microparticles [9].

Chitosan was selected as the polymeric component to prepare composite scaffold. It is a natural hydrophilic polymer, it allows the formation of a thermogelling polymer solution in the presence of glycerol phosphate disodium salt (βGP). The lyophilized formulation, obtained combining the thermogelling polymer solution with the bovine bone substitute (BBs) granules (Orthoss®), has been designed, prepared and characterized in previous works by the same authors [10,11]. The composite scaffold has been proposed for bone regeneration, as filler system with osteogenic and osteoconductive properties.

Starting from this background, the present work deals with the formulation study of a 3D composite scaffold loaded with gentamicin loaded microparticles (3D-DDS) capable to prevent or treat the osteomyelitis achieving gentamicin prolonged release and support the regeneration of new tissue. In these terms, the scaffold loaded with gentamicin loaded microspheres is here proposed as a biodegradable drug delivery system for the local delivery of antibiotic to bone, and promoting bone regeneration ad composite scaffold as scaffold for tissue regeneration. The study involves the preparation of gentamicin loaded 3D-DDS and its complete characterization in terms of physico-chemical and bactericidal properties as well as stability.

2. Materials and methods

2.1. Materials

Gentamicin sulphate (Gentamicin C1 C21H43N5O7, Mw 477.6 g/mol, Gentamicin C2 C20H41N5O7, Mw 463.6 g/mol, Gentamicin C1a $C_{19}H_{39}N_5O_7$, Mw 449.5 g/mol), was from Sigma Aldrich. 5050 DLG 4C PEG 1500 (Mw 47 kDa PEG % 51) was from Lakeshore Biomaterials (Birmingham, AL, USA), PVA (Mw 85–124 KDa 87–89% hydrolized), methylene chloride, dimethyl sulfoxide, sodium chloride, ninhydrin, Mw 178,14 g/mol, glycerol 2-phosphate disodium salt hydrate (β-GP) 216.16 Mw, were from Sigma Aldrich, (Sigma Aldrich Milan, Italy).

Orthoss® granules were supplied by Geistlich Surgery (Geistlich Pharma AG, Wolhusen Switzerland), with size and size distribution of d_{10} 141.437 mm, d_{50} 721.029 mm and d_{90} 1340.246 mm, Span value 1.663. Chitosan chloride CL213, Protasan CL213, deacetylation degree 82%, hydrochloric acid content 10–20%, Mw 350 KDa was purchased from Pronova Biomedical (Pronova Biomedical, Norway). Human adult dermal fibroblasts as primary cells were purchased from International PBI, (PBI, Milan, Italy). Distilled water was filtered through 0.22 μm membrane filters (Millipore Corporation, Massachusset, USA) before use. Unless specified, all other solvents and reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of gentamicin microparticles

Microparticles preparation was performed as reported in a previous work by the authors [9]. Microparticle formulations were prepared by double emulsion with evaporation of solvent. Briefly, primary emulsion was prepared by homogenizing of 7.5% w/v gentamicin aqueous solution (1 ml) into 9 ml of 5050 DLG 5C PEG1500 polymer solution (15% w/v) in methylene chloride, at 17,500 rpm for 2 min. The obtained emulsion was dispersed into a secondary aqueous continuous phase containing PVA (2% w/v) and