We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



8

PLGA-Alendronate Conjugate as a New Biomaterial to Produce Osteotropic Drug Nanocarriers

Rosario Pignatello
Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Catania
Italy

1. Introduction

Targeting bone tissue to treat specific diseases, such as primary tumour or bone metastases from peripheral malignancies is a current and pressing research field for modern pharmaceutical technology, and nanomedicine in particular. Bone is the third most common site of metastasis and the incidence of bone metastases in patients died of cancer is reported to be around 70% [1,2]. Primary carcinomas of the lung, breast, prostate, kidney, and thyroid may develop skeletal metastases. In children affected by disseminated neuroblastoma bone metastases are also highly frequent.

Tumour osteolysis is responsible for pathologic fractures, intractable pain, nerve compression syndrome, and severe hypercalcemia. In addition, patients with bone metastases may have neurologic impairment from spinal lesions, anaemia, and complications exacerbated by immobilization. Bone matrix resorption is minimally due to direct cancer cell activity. In fact, bone-colonizing tumour cells also stimulate osteoclast-mediated bone resorption via the secretion of potent osteolytic agents [3]. The increased bone resorption that follows releases bone-derived growth factors into the extracellular milieu and systemic circulation, thereby further enhancing bone resorption, promoting tumour growth, and altering the tumour microenvironment. Vice versa, metastatic cancer cells are largely influenced by messages embedded within the bone matrix [4]. Under the influence of bone microenvironment, tumour cells proliferate and interact with osteoblasts and osteoclasts leading to lytic or sclerotic lesions.

Clinical management of metastatic bone disease is really hard. The probability of long-term survival decreases dramatically in patients with skeletal metastases. Current treatments are based both on systemic therapy (chemotherapy, immunotherapy, hormone therapy) [5,6] and local therapy (surgery and radiotherapy) [7,8].

The restoration of functionality and mobility, along with pain relief can improve patients' quality of life, but is unable to affect the negative progression of the disease. Because of the unique characteristics of tumours growth within bone tissues, conventional therapeutic strategies often lack efficiency in cure bone metastases. Moreover, most of the drugs used for the adjuvant therapy of osteolytic metastases interfere with signalling that induce osteoclast differentiation and osteoclast-mediated bone resorption, but they are not effective on tumour growth in the bone and their use is obviously associated with heavy side effects.

A number of drugs are effective for the treatment of bone tumours, but their systemic delivery is inevitably associated with significant side effects and lack of targeting. Thereby, targeting specific biochemical patterns inside bone cancer areas may theoretically provide a mean to improve the efficacy and reduce the required dose of anticancer drugs.

1.1 Bone targeting strategies

Development of osteotropic drug delivery systems (ODDS) is therefore an appealing issue in the wide field of innovative pharmaceutical technology. To realize an effective system, many obstacles must be overcome. Bones are covered with lining cells acting as a marrow-blood barrier; therefore, the contact of exogenous compounds to the bone surface is restricted. Furthermore, the expression of biomolecules having a specific targets, like enzymes or antigens is relatively low in mineralized tissues, thus restraining the chances for an active drug targeting.

The strategies proposed for targeting drugs to the bone can essentially be condensed in two fields: the passive targeting approach, realised by the encapsulation in or association of drugs to colloidal carriers, such as polymeric or lipid nanoparticles (NP), liposomes or dendrimers (strategy 1, **Figure 1**). Drug-loaded nanocarriers allow to achieve a selective release to some tissues, like tumours, by means of the well-known phenomenon called 'EPR effect' [9], due to the fact that all cancers are characterized by permeable (leaky) blood neovessels and an impaired lymphatic outflow. Because of their multifunctional properties, nanosized systems can carry both targeting molecules and drugs, and deliver the latter in very specific sites within the body. Another advantage of NPs is the possibility of carrying in the bloodstream poorly soluble or unstable compounds, such as peptides and proteins, preventing their premature inactivation by circulating enzymes.

However, active targeting should be in some case more useful to reach effective drug concentrations in specific tissues and organs. Thus, other approaches have also been explored in which a drug is covalently linked to a targetor moiety able to recognize the bone tissues, and selectively convey the whole compound (e.g., a prodrug or a polymeric conjugate) to the site of action (strategy 2, **Figure 1**) [10].

Since bones are made of a mineralized matrix, a logical solution to the problem of bone targeting would be the development of delivery systems that possess affinity for hydroxyapatite (HA) via osteotropic molecules, such as bisphosphonates (BP) [11,12]. BP are synthetic, non-hydrolysable compounds structurally related to pyrophosphate. The P-C-P structure of BP is responsible for the ability of binding divalent ions, such as Ca^{++}, and thereby for the high affinity to HA [13]. Upon administration, BP are hence rapidly cleared from the bloodstream and bind to bone mineral surfaces at sites of active bone remodelling, like the areas undergoing osteoclast resorption [13].

BP are the most effective antiresorptive agents for the treatment of bone diseases associated to an increase in the number or activity of osteoclasts, including tumour-related osteolysis and hypercalcemia [14]. Moreover, BP are also able to reduce the survival, proliferation, adhesion, migration, and invasion of tumour cells [15, 16] and to inhibit angiogenesis in experimental and animal tumour models [17]. Since HA crystals are only present in 'hard' tissues, like teeth and bones, conjugation to BP can represent a valid strategy for selectively deliver bioactives to the bones.

The scientific literature presents many papers and patents exploring such approach, sometimes carrying original ideas or integrating together different chemical and technological pathways. In particular, osteotropic drug delivery systems (ODDS) have been proposed some years ago as a possible mean to impart to drugs an affinity to bone tissues

[11, 12, 18, 19]. Bisphosphonates (BP) have been for instance conjugated to small drugs [20, 21], and proteins [22] with the aim at optimizing the treatment of osteoporosis, osteoarthritis, and bone cancer, and to radiopharmaceuticals to obtain novel agents for bone scintigraphy [23]. The conjugation of bisphosphonates to polymer backbones has been studied as a mean for bone targeting [24,25]. Recently, cholesteryl-trisoxyethylene-bisphosphonic acid (CHOL-TOE-BP), a new tailor-made BP derivative, has been used as a bone targeting moiety for liposomes [26]. In other works, the amino-bisphosphonate drug alendronate (ALE; Fosamax®) was co-conjugated to HPMA polymer backbones together with an anticancer agent. Thereby, passive targeting was achieved by extravasation of the nanoconjugates from the tumour vessels via the EPR effect, while active targeting to the calcified tissues was achieved by ALE affinity to HA [27].

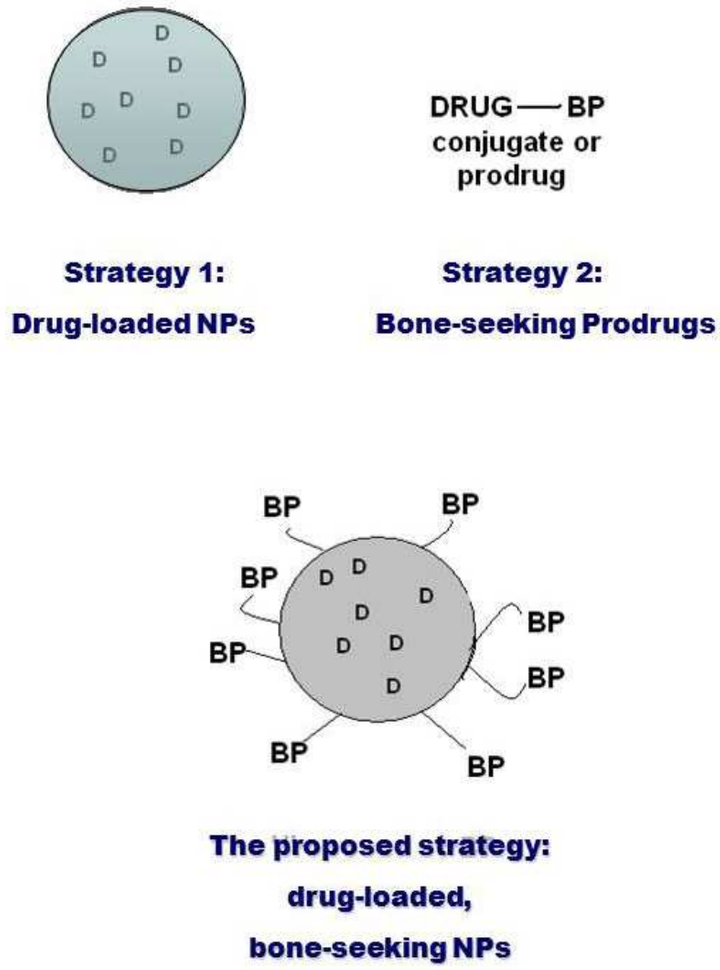


Fig. 1. The strategies described in the literature to achieve targeting to bone tumours: 1) an anticancer drug (D) is encapsulated/loaded into a nanocarrier (e.g., polymeric or lipid nanoparticles, liposomes, micelles, etc.) and passive targeting is expected (EPR effect); 2) a biologically active molecule is covalently linked to an osteotropic targetor moiety (e.g., a bisphosphonate, BP); 3) nanoparticles are made by an osteotropic polymer (e.g., a polymer-BP conjugate) and then loaded with a bioactive agent: in this way, both passive and active targeting possibilities can be achieved.

1.2 The scientific and technical rationale for a new targeting strategy

Targeted DDS should be preferable over drug-BP conjugates due to different factors, such as drug protection from biodegradation in the bloodstream, transport duration, and drug-payload. Thereby, in a recent and partially still on-going research, the two strategies depicted in **Figure 1** have been merged, leading to an innovative solution for active targeting of drugs to the bone. The working hypothesis was to realize a biocompatible nanocarrier showing high affinity to bone (i.e., an osteotropic nanocarrier), which can be loaded with different classes of drugs active against bone diseases, such as anticancer, anti-angiogenic, antibiotics, or anti-osteolytic agents.

To this aim, in a first step a new polymeric biomaterial showing osteotropic properties was produced through the conjugation of a poly(lactide-*co*-glycolide) (PLGA) to the BP agent alendronate (ALE). PLGA copolymers are diffusely used biocompatible and biodegradable materials for controlled drug release systems [28], including anticancer agents, and they have also been recognized as GRAS by US FDA. In this study, a copolymer made of 50:50 polylactic-*co*-glycolic acid was used (Resomer® RG 502 H; **Figure 2**) because of the presence of free carboxyl end groups susceptible to chemical derivatisation. The typical weight composition of this copolymer allows it to remain in the human body enough to let the bound BP moiety to recognize the bone proteins.

ALE (4-amino-1-hydroxybutyldiene-1,1-phosphoric acid, **Figure 2**) is an amino-BP, approved for the treatment and prevention of osteoporosis, treatment of glucocorticoid-induced osteoporosis in men and women, and therapy of Paget's disease of bone [29]. ALE was selected among BP agents because of some peculiar properties: a) the presence of a free amine group, able to create a stable covalent link with the carboxyl group present in the used PLGA; b) such amine group is not essential for the interaction of ALE with HA, and thus for its pharmacological effects, but exerts only a supportive role [30]; c) the possibility of easily converting the commercial sodium salt of ALE into its free acid form. The choice of an amide bond between the targeting BP and the polymer was also driven by the known relatively high resistance to enzymatic hydrolysis in plasma of this linkage, that should ensure the intact conjugate to reach the target (bone) tissues.

The haemo- and cytocompatibility of the PLGA-ALE conjugate was confirmed *in vitro*. Therefore, a nanoparticle system (NP) was produced starting from this new biomaterial. The NP were studied for their technological properties, as well as for their biocompatibility. Finally, PLGA-ALE NP were then loaded with a model cytotoxic drug, doxorubicin (DOX) and tested *in vitro* and *in vivo*.

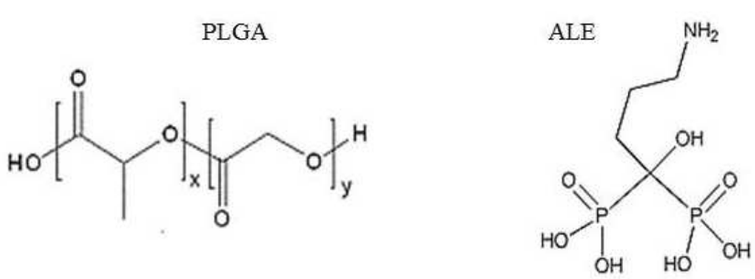


Fig. 2. Schematic structure of Resomer® RG 502 H (PLGA) and alendronic acid (ALE).

2. Synthesis and characterization of the PLGA-ALE conjugate

Polyl(D,L-lactic-*co*-glycolic acid) (50:50) containing a free carboxylic acid end group [Resomer® RG 502 H; inherent viscosity: 0.16-0.24 dl/g (0.1% in chloroform, 25°C)] was used (Boehringer Ingelheim, Ingelheim am Rhein, Germany). Sodium alendronate was converted into the acid form by treatment with aqueous acetic acid and lyophilisation. The conjugate was synthesized by two alternative methods, i.e., carbodiimide-assisted direct conjugation or preparation of an activated intermediate through N-hydroxysuccinimide [31]. In the first case, a solution of Resomer® RG 502 H in DMSO and dichloromethane (DCM) (1:1) was activated at 0°C for 2 h by N'-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDAC), in the presence of 1-hydroxy-benzotriazole (HOBt) and triethylamine. Alendronic acid was dissolved in DMSO and added to the reaction mixture, which was stirred for 2 h at 2°C and then at r.t. for 8 h. The solvent was partially removed under vacuum and the remaining solution was purified by dialysis water (CelluSep H1 MWCO 2000; M-Medical s.r.l., Cornaredo, Italy). In the alternative procedure, the PLGA was previously activated with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide in anhydrous dioxane at 15°C under stirring for 3 h [32]. The formed dicyclohexylurea was filtered off and the solution was poured in anhydrous diethyl ether. The solvent was decanted and the oily residue was purified by dissolution in anhydrous dioxane and precipitation with anhydrous diethyl ether (3 times), and finally dried in vacuo. A solution of NHS-PLGA in anhydrous DMSO was treated with triethylamine and sodium alendronate under stirring at r.t. for 12 h. At the end of the reaction the solvent was partially removed under vacuum and the remaining solution was purified by dialysis. The dialysed samples were frozen into using liquid nitrogen and lyophilised. Both methods resulted in similar production yields (70-75%) and purity of the final conjugate, therefore the first method could be better proposed as more direct and simple. The chemical structure of the PLGA-ALE conjugate was confirmed by MALDI-TOF MS and ^{1}H-NMR; analytical details are available [31].

In the view of using the PLGA-ALE copolymer to prepare bone-targeted NPs, the conjugate was evaluated for blood and cyto-compatibility, to individuate any negative effect which might have precluded any further biological investigation. Haemolysis was evaluated because erythrocytes are among the first cell lines that come into contact with injected materials. Experimental results did not show haemolytic effects of the conjugate; the plasmatic phase of coagulation was measured by the activated partial thromboplastin time (APTT) and prothrombin activity; they respectively evaluate the intrinsic and extrinsic phases of coagulation. In basal conditions, prothrombin activity was 140.2 ± 2.5 and APTT was 35.6 ± 0.3. In plasma incubated with PLGA-ALE at different dilutions, prothrombin activity and APTT were not significantly different from the plasma incubated with PBS (**Figure 3A** and **3B**). DMSO at the same dilutions did not affected either prothrombin activity or APTT.

In the bloodstream, nanoparticles come in contact with endothelium before passing through the vessel wall and reaching tissues. Therefore, the effect of PLGA-ALE on endothelial cells was tested to verify the lack of cytotoxicity. As expected, PLGA-ALE was not cytotoxic for human umbilical vein endothelial cells (HUVEC), as proven by the neutral red test (**Figure 3C**). Absence of cytotoxicity was also shown in cultures of human primary trabecular osteoblasts (BMSC) (**Figure 3D**).

In conclusion, PLGA-ALE conjugate did not cause either haemolysis on human erythrocytes, or alterations of the plasmatic phase of coagulation or cytotoxic effects on endothelial cells and trabecular osteoblasts [31].

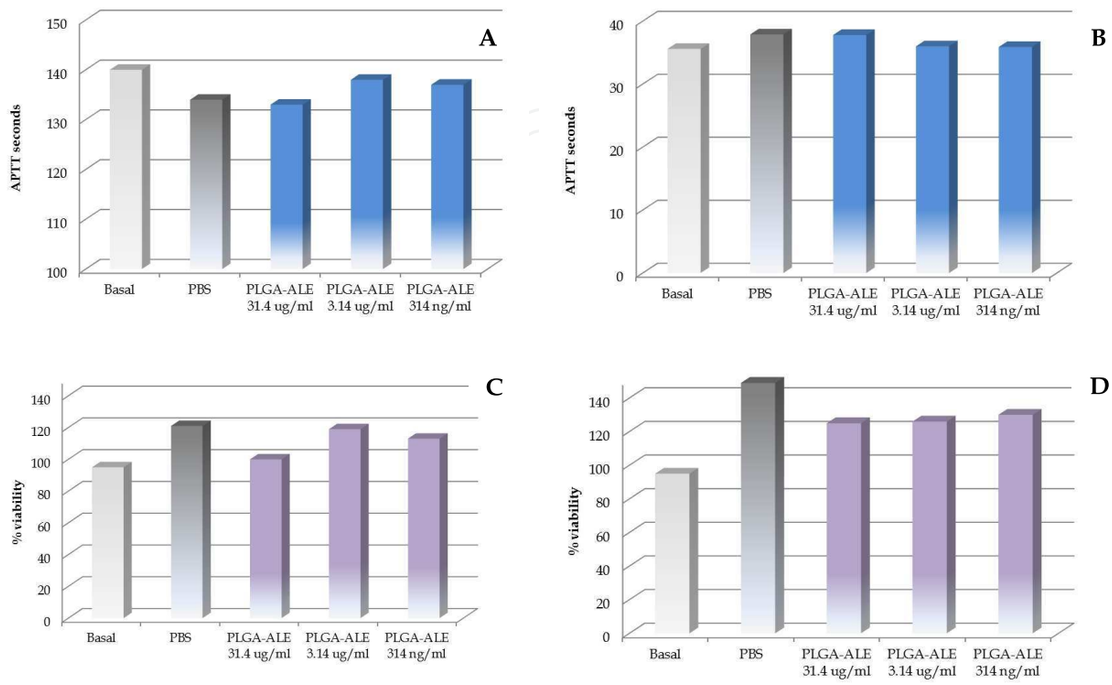


Fig. 3. Biocompatibility results of the PLGA-ALE conjugate: (A) mean prothrombin activity and (B) APTT of human plasma incubated with different concentrations of the conjugate; (C) viability of human umbilical vein endothelial cells (HUVEC) and (D) human primary osteoblasts from trabecular bone, respectively, after incubation with the conjugate. No haemolytic activity was given by the tested compound (not shown) (adapted from ref. [31]).

3. PLGA-ALE nanoparticles

NP were produced by the PLGA-ALE conjugate by a nanoprecipitation method, using an opportunely adapted emulsion/solvent evaporation technique [33]. The conjugate was dissolved in acetone, DMSO or their 1:1 mixture (*v:v*). The organic solution was added drop wise into phosphate buffered saline (PBS), pH=7.4, containing Pluronic F68. After stirring at r.t. for 10 min, the solvent was partially removed at 30 °C under reduced pressure and the concentrated suspension was purified by dialysis against water.

In **Figure 4** the size analysis of the formed systems, obtained by photon correlation spectroscopy is illustrated. DMSO was used in the NP production due to the low solubility of the conjugate in acetone (from which low production yields were achieved), but it did not appear to be an ideal solvent; in fact, NP obtained from pure DMSO solution showed a higher mean size with respect to those obtained using an acetone/DMSO mixture. These NP showed a homogeneous distribution, with an average size of 198.7 nm and a polidispersity index of about 0.3. However, in all cases mean sizes between 200-300 nm were obtained, an interesting feature for a further development of these systems as injectable drug carriers.

Also a dialysis method using a DMSO solution of the conjugate was attempted to prepare the NP [34], but it resulted in much larger particles (around 400 nm) (not shown).

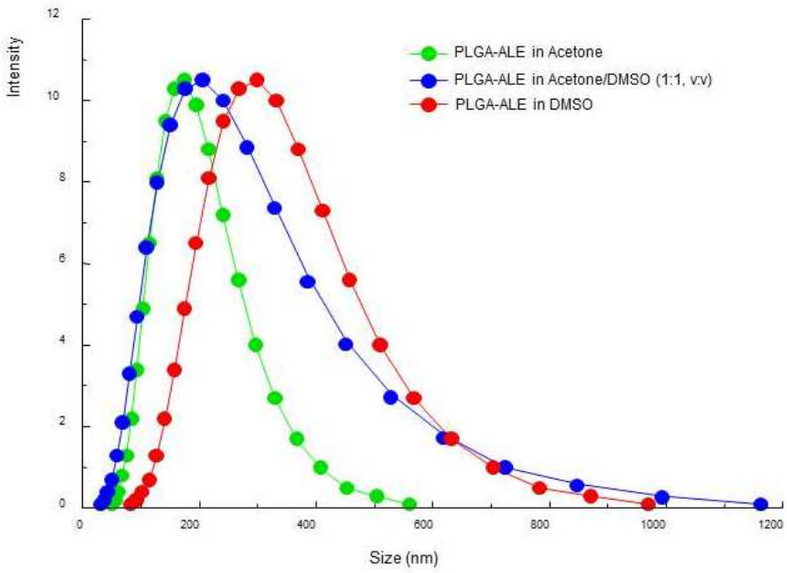


Fig. 4. Size distribution patterns of PLGA-ALE NP prepared using different organic phases (adapted from [31].

The PLGA-ALE NPs showed a net negative surface charge (ζ-potential of –38 mV), close to the value given by the NPs obtained from pure PLGA (-41.8 mV). SEM analysis revealed spherical particles with a smooth surface (**Figure 5**). The system was also shown to be sterilisable by gamma irradiation at 10 kGy, showing only minimal particle size changes [33]. This finding prospects the possibility of using sterilized NPs for further *in vivo* studies of the system.

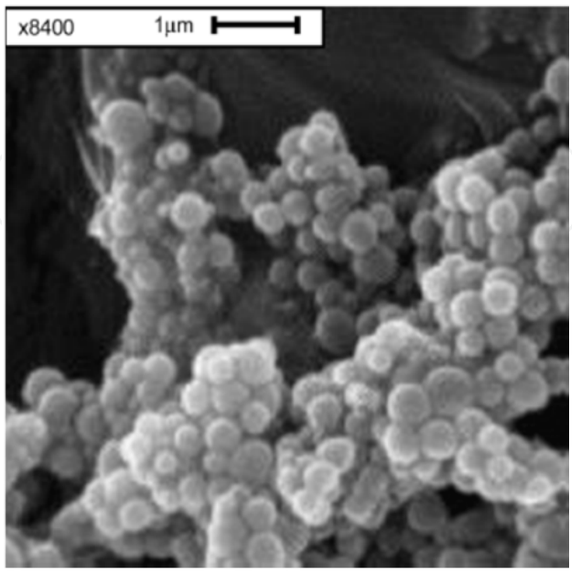


Fig. 5. SEM picture of PLGA-ALE NPs produced in 1:1 acetone/DMSO.

To assess the affinity for HA, PLGA-ALE and PLGA NPs were loaded with a lipophilic probe (Red Oil O) and incubated with two different HA concentrations (1 and 5 mg/ml) for 15 or 30 min. PLGA-ALE NPs showed a relative increase of affinity towards the phosphate salt compared to pure PLGA NPs (**Figure 6**). The affinity increased with the incubation time and was proportional to the concentration of HA in the suspension. This latter observation would suggest that some form of chemical interaction occurred between PLGA-ALE and HA, reinforcing the mere physical absorption of the phosphate on NP surface, which justifies the affinity already measured for pure PLGA NPs.

The biocompatibility profile of PLGA-ALE NPs was assessed by means of several *in vitro* assays, able to demonstrate their effects on biological systems which use to come in contact with a material injected into the body [33, 35, 36]. The use of NPs for drug delivery necessitates an accurate assessment of their biocompatibility [37, 38]. For their nanoscale size, NPs may have a reduced blood compatibility in comparison with the starting material: even if the biocompatibility of a macromolecule is well-established, the enormous increase of its surface when in the form of NPs may bring on negative effects that are not given by the bulk material.

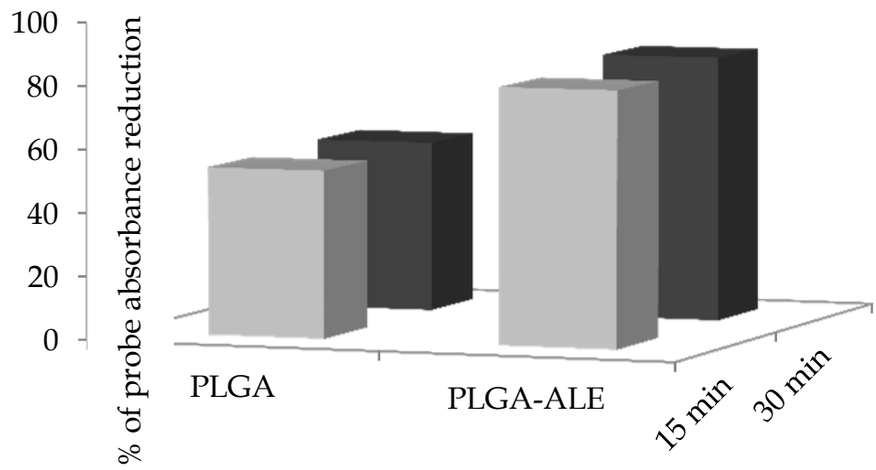


Fig. 6. Affinity of PLGA-ALE and PLGA NPs for hydroxyapatite (HA). Oil Red O-loaded NP suspensions were incubated at r.t. for either 15 or 30 min with an aqueous suspension containing 5 mg/ml of the phosphate salt. Results are expressed as the percentage decrease of light absorbance at 523 nm with respect to the corresponding NPs incubated without HA [*cf*. ref. 33].

3.1 Biocompatibility studies

Blood-biomaterial interactions are complex events that involve erythrocyte and leukocyte damage, and activation of platelet, coagulation, and complement. Damage of red blood cells, as well as complement activation may favour haemolysis and other forms of general toxicity. Analogously, NPs should not activate platelets and the plasmatic phase of coagulation, nor damage endothelial cells to avoid thrombogenesis [39, 40]. In the meantime, they should not reduce the levels of the plasmatic factors of coagulation, to prevent haemorrhagic accidents. Finally, with the aim of bone targeting, when NP will

reach bone tissues they should inhibit osteoclast activity without altering osteoblasts. Therefore, a preliminary evaluation of NP biocompatibility has been necessary before their loading with actives.
The PLGA-ALE NP at different concentrations did not show any haemolytic effects towards a suspension of human erythrocytes. The percentage of haemolysis was in fact similar to the erythrocytes incubated with PBS (Table 1).

Sample	NP concentration	% of haemolysis
PLGA-ALE NP	56 μg/ml	0.001±0.165
	5.6 μg/ml	0.289±0.320
	560 ng/ml	0±0.272
	56 ng/ml	0.356±0.309
	5.6 ng/ml	0.167±0.191
	0.56 ng/ml	0±0.155
PBS	-	0
Saponin	-	132.72±2.74
Distilled water	-	100

Table 1. Hemolytic activity (arithmetic mean ± S.E. of 6 experiments) of PLGA-ALE NPs. Their concentration was expressed as the amount of ALE in each sample.

As a further proof of their compatibility with blood components, at all the tested concentrations PLGA-ALE NPs did not induce any significant effect either on the total leukocyte number or on their subpopulations percentage. Analogously, the NPs did not cause platelet adhesion or activation (release reaction), as assessed by the platelet factor 4 measurement (**Figure 7**), all process that may induce thrombotic phenomena after NP injection [33].

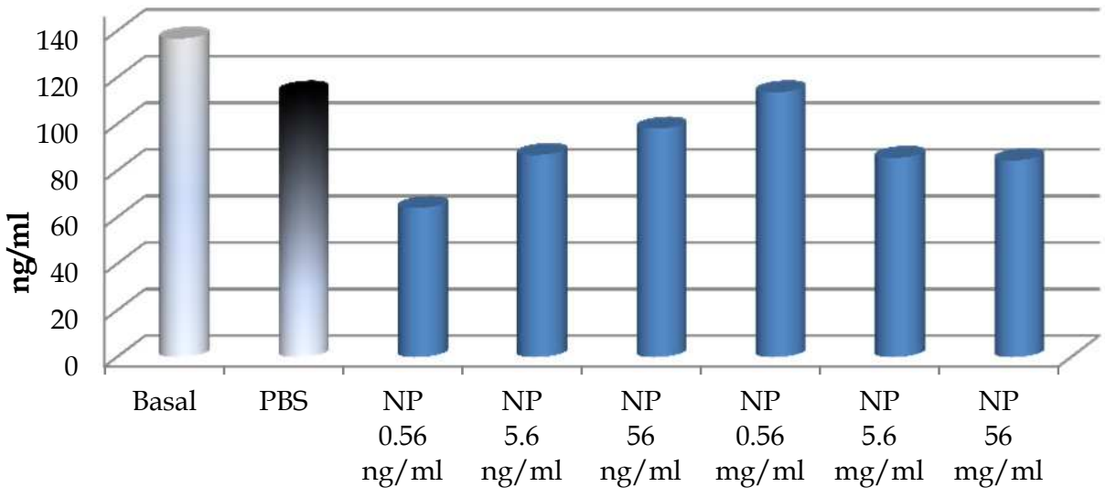


Fig. 7. Platelet factor 4 concentration after incubation with PLGA-ALE NP.

To assess the lack of alterations of blood proteins, we evaluated the effects of PLGA-ALE NPs on the plasmatic phase of coagulation and on complement. A decrease of the coagulation factors levels favours haemorrhage, while an increase of their activity may induce thrombotic phenomena. In our experiments, only the highest tested concentration of NPs caused a decrease of the prothrombin activity, while all the lower concentrations increased it, with changes however always ranging within normal physiological values (**Figure 8**). Since APTT (i.e., the intrinsic and the common phase of coagulation) was also not significantly affected by the NPs (not shown), the observed changes could be due to an alteration of Factor VII of the extrinsic pathway of coagulation. Probably, Factor VII was adsorbed on the NPs surface at high concentration and was less available for coagulation; conversely, lower concentrations of PLGA-ALE NPs could activate factor VII, in a way similar to the effect of tissue factor. Another hypothesis could involve the activation of Factors XII and XI by the NPs, similarly to silica gel or glass (*cf.* [33] for more details).

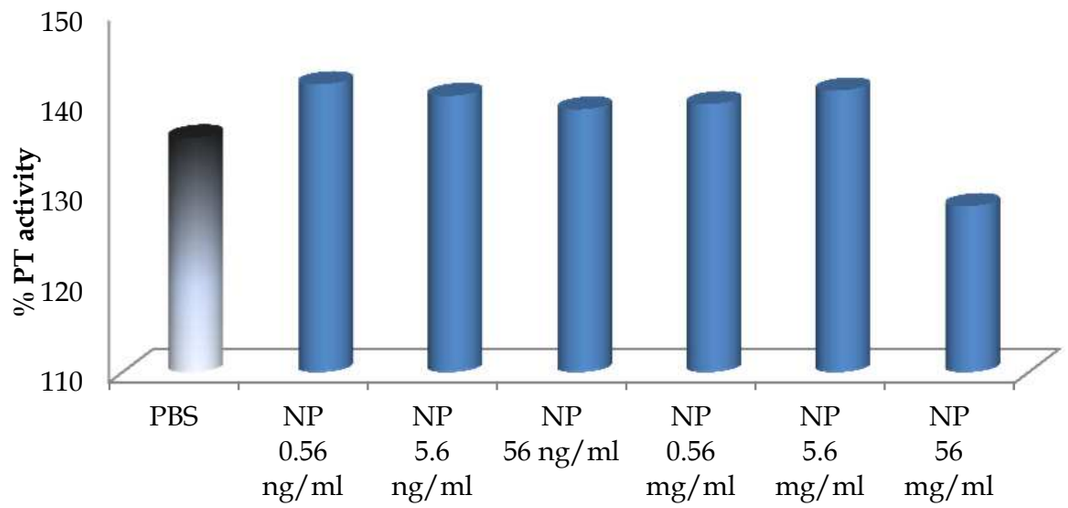


Fig. 8. Protrombin activity induced by different PLGA-ALE NP concentrations.

Activation of complement induces the production and release of small biologically active peptides. C3a and C5a are chemo-attractive for leukocytes and favour their aggregation. Furthermore, C5a induces the adhesion of granulocytes and monocytes to the endothelium, their migration into the external tissues, enzyme release and production of pro-coagulant or platelet aggregating compounds. C3a and the C5b-9 complex can directly activate platelets. Therefore, complement activation should not be considered as a strictly local phenomenon, but results also in systemic effects. Complement activation may occur by the classical and the alternative pathways. In the classical pathway, the protein C1q recognises activators (usually immune complexes) and binds to them. The activation via the alternative pathway starts by the binding of C3b to the activator surface (such as microbial polysaccharides or lipids or surface antigens present on some viruses, parasites and cancer cells) and then follow the same events of the classical pathway. NPs predominantly activate the complement through the alternative pathway.

In our experiments PLGA-ALE NPs did not activate complement by none of the two pathways, as shown by a non-significant different complement consumption from PBS (**Figures 9A** and **9B)**. Conversely, the positive control zymosan induced a 70% complement consumption, twice the amount consumed by PBS. Also the Bb fragment, which is produced during the complement activation by the alternative pathway, was not significantly affected by the PLGA-ALE NPs (not shown).

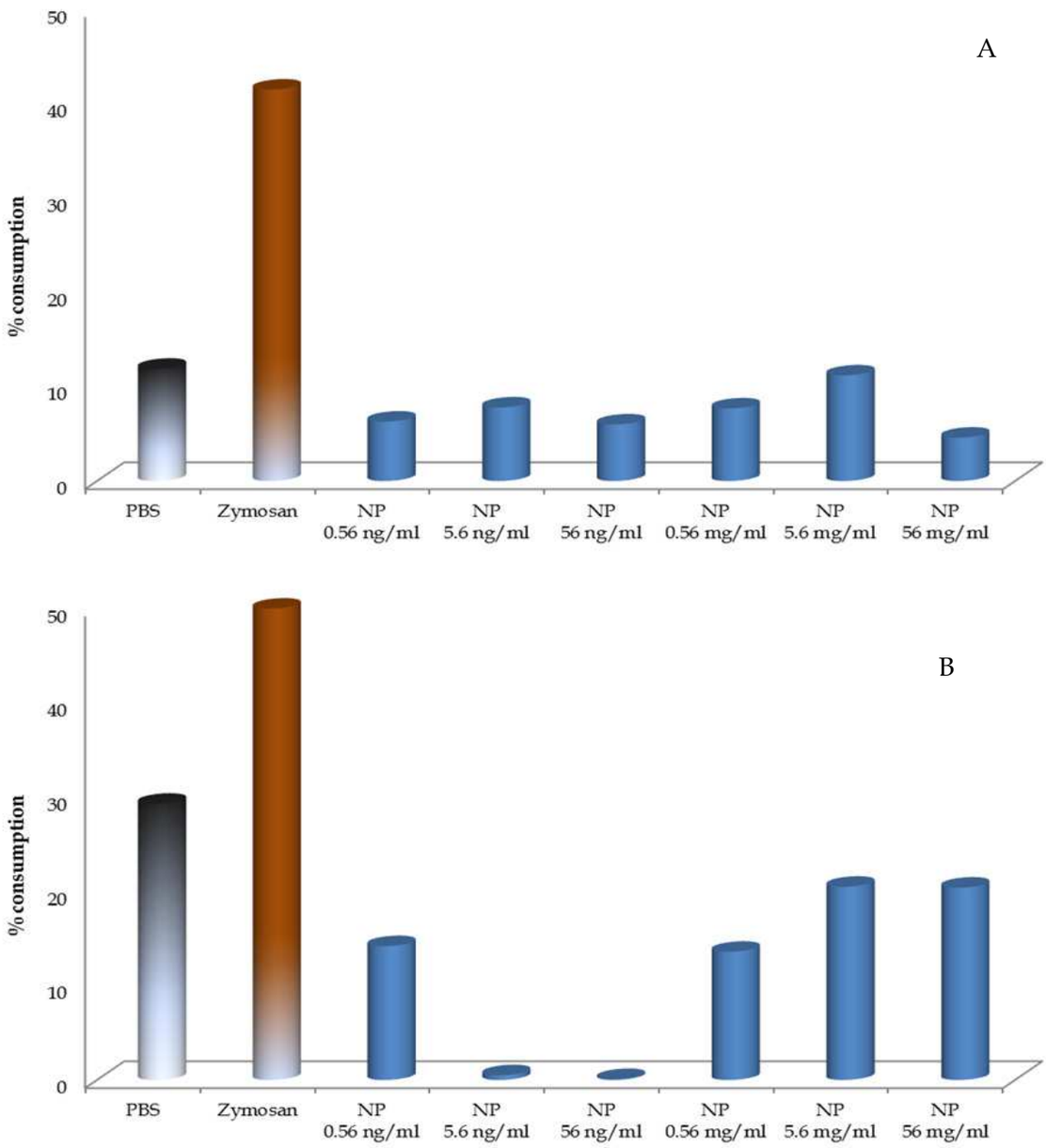


Fig. 9. Percentage consumption of human serum complement activity via the classical pathway (A) or the alternative pathway (B), after incubation with the PLGA-ALE NPs (adapted from ref. [33]).

In the bloodstream, NP come rapidly in contact with vessel endothelium before passing to the outer tissues; therefore, absence of damage to endothelial cells must be ensured. Moreover, bone oriented NPs should not affect the vitality and function of normal osteoblasts. The cytotoxicity of the prepared PLGA-ALE NPs was excluded on both endothelial (HUVEC) cells and osteoblasts derived by bone marrow stromal cells (BMSC). Cell viability was always higher than 80% upon 24 h-exposure to the various concentrations of NPs or to PBS. Phenol, used as a positive control, reduced the cell viability to 19.0% (HUVEC) and to 27.5% (BMSC) (**Figure 10)**.

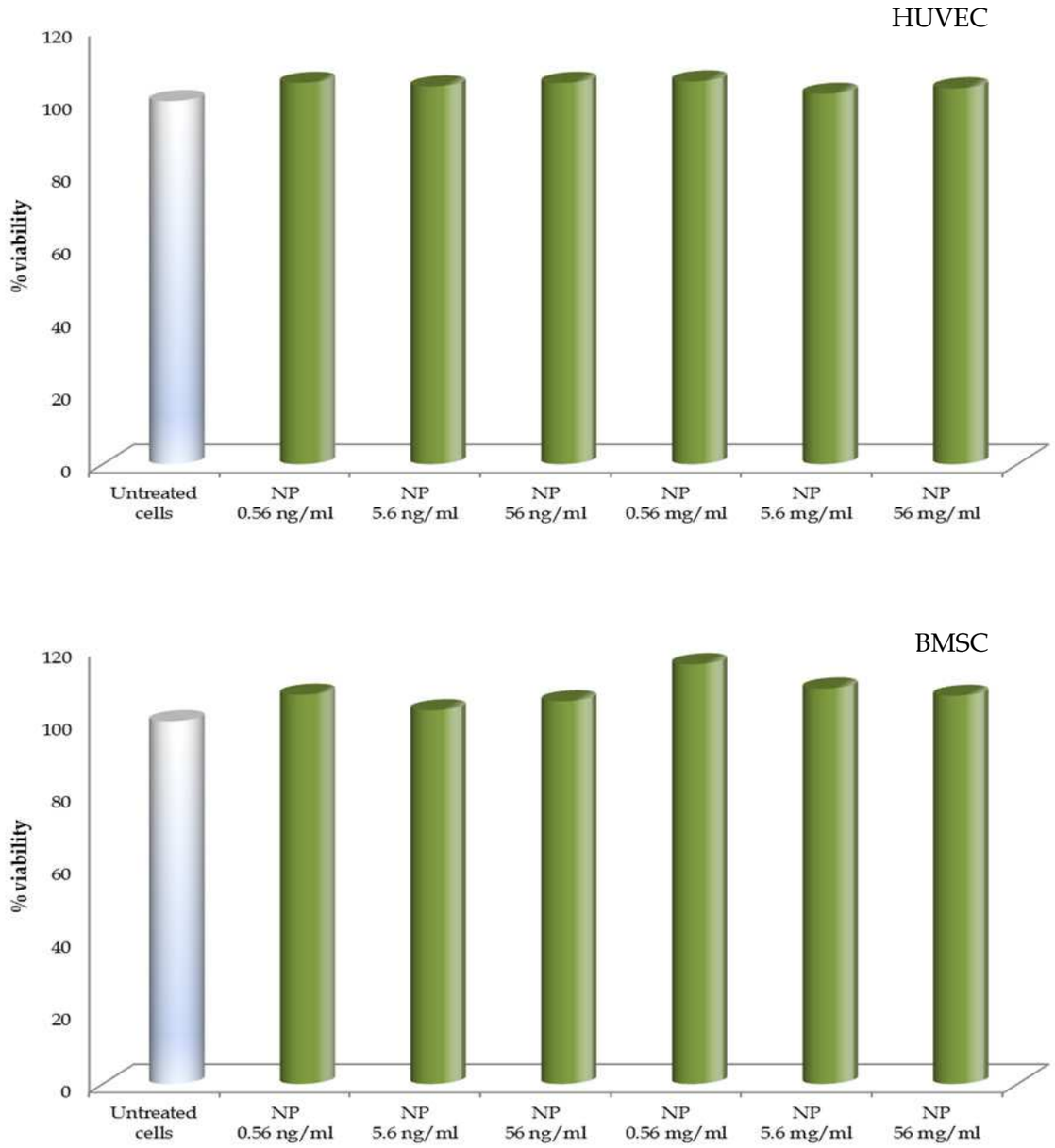


Fig. 10. Viability of endothelial (HUVEC) cells and osteoblasts (BMSC) exposed to various concentrations of PLGA-ALE NPs (adapted from ref. [33]).

In conclusion, PLGA-ALE NPs did not affect platelets, leukocytes and complement, did not induce haemolysis and did not exert cytotoxic effects on endothelial cells and osteoblasts [33].
To assess if the PLGA-ALE NPs are able to retain the antiosteoclastic properties of the bisphosphonate, osteoclast cultures obtained from human peripheral blood mononuclear cells (PBMC) were incubated with either PLGA-ALE or pure PLGA NPs, at an equivalent concentration of 0.64 μM or 6.4 μM of ALE; free ALE was tested as a positive control. Experiments showed that ALE retained the ability of inhibiting the osteoclast-mediated degradation of type I human bone collagen, determined a dose-dependent reduction of osteoclast number, and induced apoptosis in osteoclast cultures, also when conjugated with the copolymer PLGA and in the form of NPs [41]. Interestingly, pure PLGA NPs also showed similar effects, and this can be considered an useful phenomenon in the view of the overall aim of this study: since the conjugation of ALE to PLGA, and the resulting NP formation was aimed at targeting antitumor drugs to osteolytic bone metastases, the additional antiosteoclastic effect observed for polymer-bound ALE and also of PLGA could even contribute to the inhibition of the associated osteolysis.

4. Drug-loaded nanoparticles

Doxorubicin (DOX) is an anticancer agent of wide clinical use, from leukaemia and Hodgkin's lymphoma to symptomatic metastatic breast cancer, from neuroblastoma to many other cancers (prostate, thyroid, bladder, stomach, lung, ovary). Its therapeutic applications are nevertheless limited by the strong cardiac and bone marrow toxicity. DOX effectiveness has been greatly improved when specific targeting at the tumour sites has been achieved, for instance by loading the drug into liposomes (e.g., Caelyx®/Doxil®) [42, 43].
DOX was loaded in the previously described PLGA-ALE NPs and the anti-tumour effect of the carrier was assessed *in vitro* and *in vivo* [44]. In a first instance, the intracellular accumulation and distribution of DOX-loaded NPs was assessed by fluorescence and confocal microscopy, in comparison with free DOX. A panel of potential target cells was used in these experiments: MDA-MB-231 and MCF7 breast adenocarcinoma cell lines, Saos-2 and U-2 OS osteosarcoma cell lines, SH-SY5Y neuroblastoma cell line, and ACHN renal adenocarcinoma cell line. The above tumour histotypes were chosen because all originate from or can metastasize to the bone.
The final localization of DOX in cell nucleus is important because of it mechanism of action [45]. We observed that the incubation of free DOX with cells resulted in its accumulation in the nuclei, while it was absent in the cytoplasm (**Figure 11**). Conversely, cells treated with DOX-loaded NPs showed also fluorescent spots localized in the cytoplasmic vacuoles. Polymeric NPs typically accumulate in lysosomal vesicles, from which the drug is released into the cytoplasm [46, 47]. Therefore, it is likely that the observed cytoplasmic fluorescence was due to the same phenomenon, and that the DOX-loaded NPs were trafficked through the endo-lysosomal compartment.
The incubation of the above cell lines with DOX-loaded PLGA-ALE NPs (48 and 72 h) gave a cell growth inhibition profile very similar to that one of the free drug [44]. To evaluate the *in vivo* activity, DOX-loaded NPs were injected into a mouse model of breast cancer bone metastases: osteolytic lesions were induced by intratibial inoculation of the human breast carcinoma cells MDA-MB-231 that can induce prominent bone metastases. Histological analysis confirmed that the NP did not cause any organ abnormality, hence they should not exert any systemic cytotoxic effect. Control mice treated with PBS developed pronounced

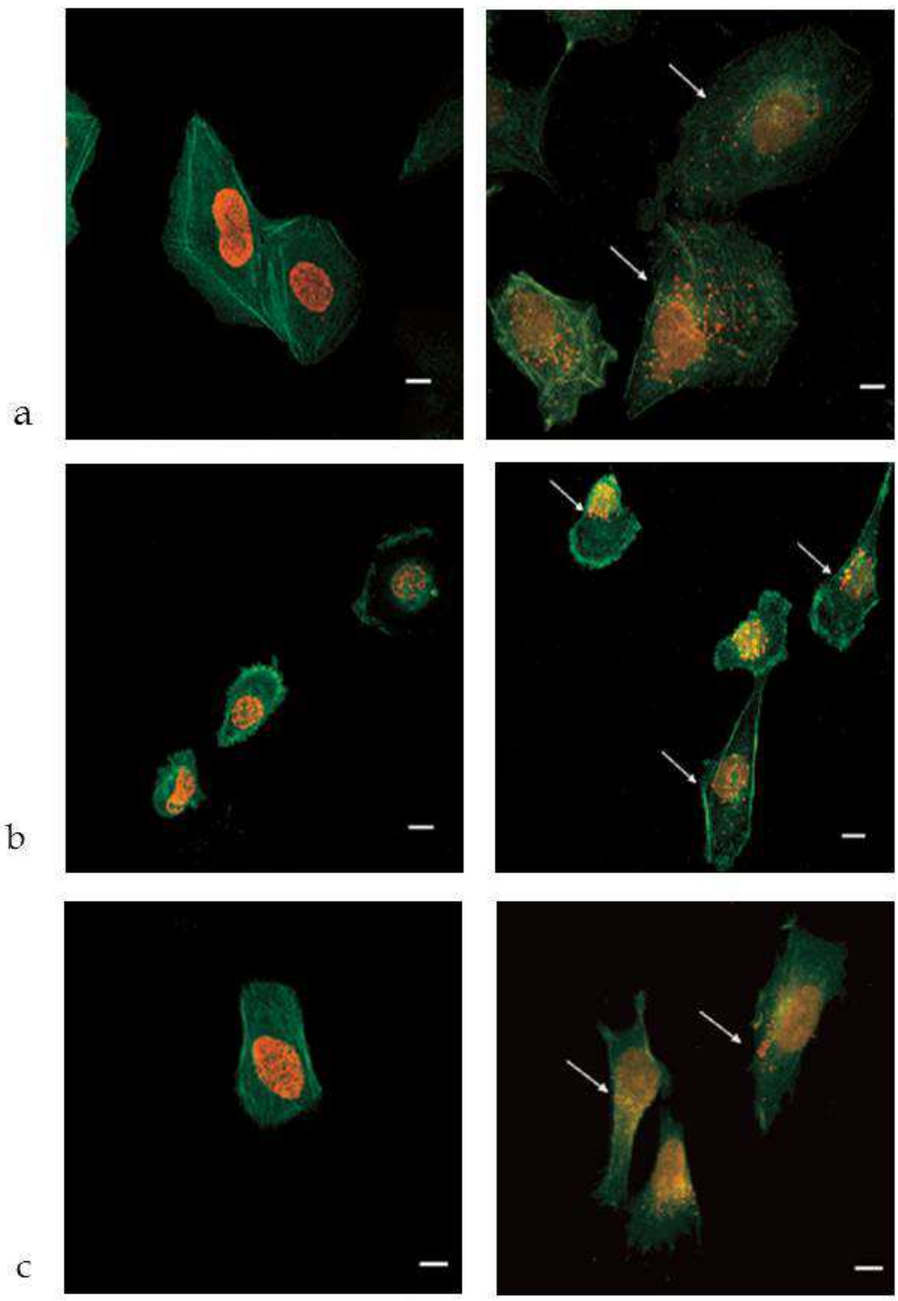


Fig. 11. Confocal analysis of cellular uptake of DOX-loaded PLGA-ALE NPs. Free DOX (left) or NP samples (right) were incubated for 24 h with U-2 OS (a), MDA-MB-231 (b), or SH-SY5Y cells (c). Cytoplasm fluorescence (red spots) was evidenced by arrows. Bars, 10 μm; magnification, 60X (adapted from ref. [44]).

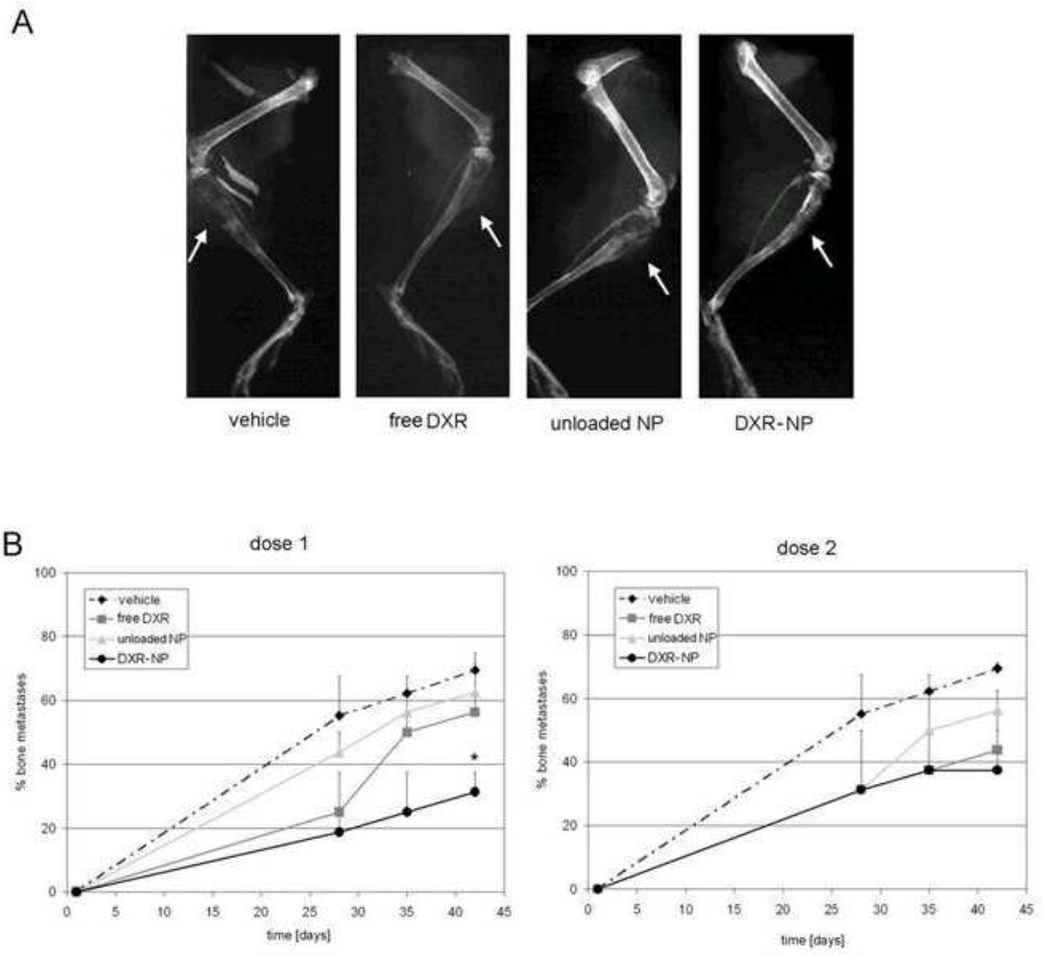


Fig. 12. Effect of DOX-loaded NPs on the incidence of osteolytic bone metastases *in vivo*. BALB/c-nu/nu mice were injected intratibially with a suspension of MDA-MB-231 cells. Mice were weekly treated for six weeks with PBS, free DOX, unloaded NPs, or drug-loaded NPs (DOX-NP) at the dose of 0.2 or 1 mg/kg. *A*, X-ray of hind limbs at day 42 of MDA-MB-231-injected mice and treated with PBS, free DOX, unloaded NPs or DOX-NP at an equivalent drug dose of 0.2 mg/kg (arrows: osteolytic areas). *B*, incidence of osteolytic bone metastases after the same treatments at the lower (left panel) and higher dose (right panel). Means ± SE, n = 8 (modified from ref. [44]).

osteolytic lesions detectable by X-ray analysis starting from the 28th day after the tumour cell inoculation (**Figure 12A**). The free drug was effective in retarding the onset of metastases and reducing their incidence compared to the animals treated with PBS or blank NPs (**Figure 12B**). However, a significant effect was observed only in the DOX-loaded NP-treated group at the dose of 0.2 mg/kg (**Figure 12B**, left panel; P= 0.028 vs. vehicle).

The extension of tumour size was significantly smaller in animal groups which received both the free drug and DOX-loaded NPs at a drug concentration of 0.2 mg/kg or 1 mg/kd (**Figure 13A**), whereas unloaded NPs were ineffective. A trend of reduction of the osteolytic areas was measured in mice treated with either free DOX, DOX-loaded NPs or unloaded NPs. However, DOX-loaded NPs induced a higher inhibition of osteolysis than unloaded

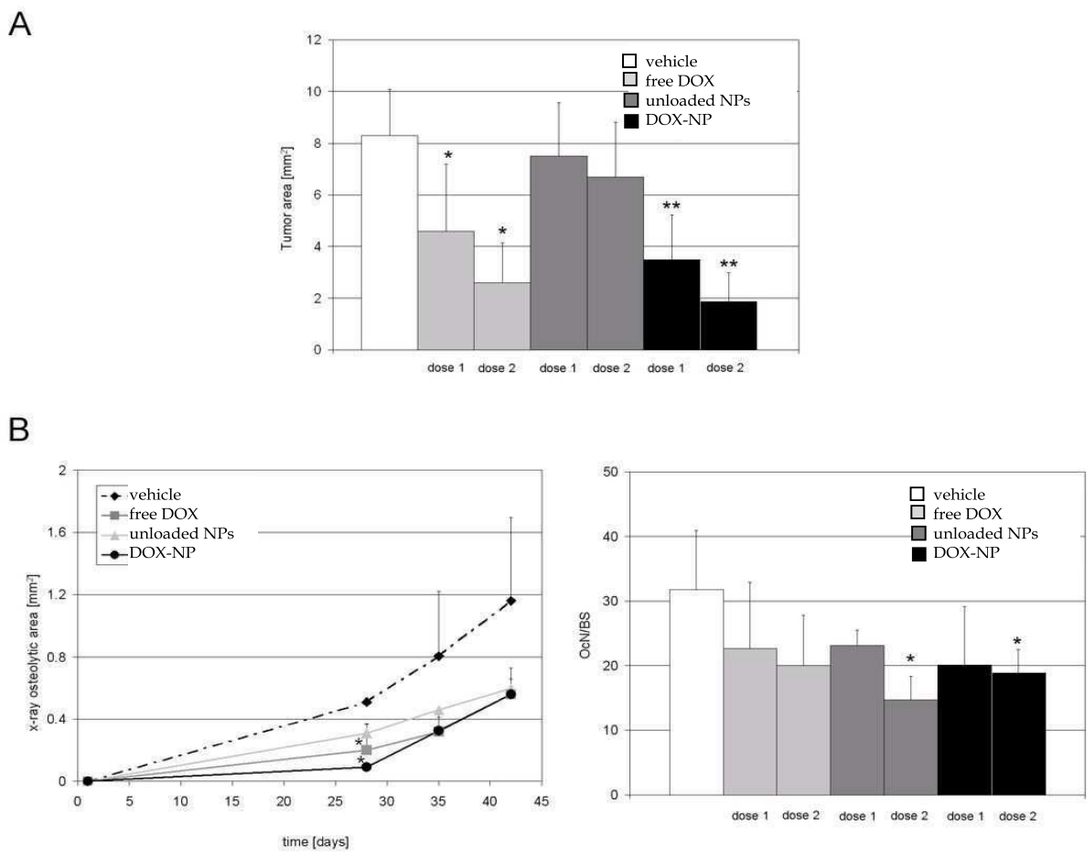


Fig. 13. Effects of DOX-loaded NPs on tumour area and osteolysis. Mice were treated with either PBS, free DOX, unloaded NPs, or DOX-loaded NPs (DOX-NP) at 0.2 mg/kg (dose 1) or 1 mg/kg (dose 2). At the end point, the extension of the osteolytic area was determined by X-ray analysis; sections of the tibiae were histologically examined to evaluate tumour area and the number of osteoclasts on the bone surface. *A*, tumour area (mean ± SD); *B*, osteolytic areas quantified on X-ray images of mice treated with dose 1 (mean± SD); *C*, number of osteoclasts found on the bone surface (OcN/BS) in TRAP stained sections of the tibiae (mean ± SD). * $P < 0.05$, ** $P < 0.005$ (modified from ref. [44]).

NPs (0.090±0.009 *vs*. 0.310±0.040 mm², $P = 0.033$); both free and NP-loaded DOX reached a similar effect at the end of the experiment (day 42) (**Figure 13B**). Similarly, the histomorphometric analysis of sections stained for TRAP activity showed a trend of reduction for the number of osteoclasts found at the bone surface by either free DOX, unloaded NPs, and DOX-loaded NPs (**Figure 13C**). However there was a significant inhibition only for unloaded NPs and DOX-loaded NPs at the highest dose (P= 0.04 and P = 0.014 *vs.* vehicle, respectively).

In conclusion, loading of DOX in the osteotropic PLGA-ALE did not affect the drug efficacy on bone metastases formation; the reduction of the incidence of metastases induced by DOX-loaded NPs was significantly higher than that allowed by the free drug. The enhanced efficacy of the drug when loaded in these NPs can be used as an indirect demonstration of

the successful delivery of DOX to the bones. The modest activity registered with unloaded PLGA-ALE NPs could be related to the inhibitory effect of ALE conjugated to PLGA on osteoclast activity, that indirectly reduced tumour expansion, and of a direct effect exerted by ALE on tumour cells [48]. Treatment of mice with both free or NP-loaded DOX significantly reduced also the tumour area.

All the above experimental findings provide *in vitro* and *in vivo* evidences of the effectiveness of a new osteotropic delivery system for DOX, and possibly other antitumour agents, in which a synergism between the antineoplastic activity of the drugs and the antiosteolytic activity of ALE can afford a better inhibition of tumour development and progression. In the meantime, loading of DOX in biodegradable and biocompatible NPs, made from a conjugate between PLGA and ALE, can allow a site-specific delivery of the drug to osteolytic areas, possibly reducing its systemic side effects. A potential tool for the development of innovative regimens for metastatic bone diseases can thus be exploited by this novel nanocarrier.

5. Acknowledgments

The wide research project described in this chapter was originally supported by a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC): "Targeting skeletal metastases by nanoscale multifunctional bone-seeking agents". Thanks are due to all the researchers from the Istituto Ortopedico Rizzoli of Bologna (N. Baldini, E. Cenni and co-workers), the University of L'Aquila (A. Teti and co-workers), and the University of Catania (Italy) (F. Castelli and co-workers), who have actively contributed to the project.

6. References

[1] Hanahan D, Weinberg RA. The hallmark of cancer. Cell. 2000;100:57.
[2] Mundy GR, Yoneda T. Facilitation and suppression of bone metastasis. Clin. Orthop. 1995;312:34.
[3] Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, Higgins LS, et al. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. Clin. Cancer. Res. 2006;12:6213s.
[4] Couzin J. Tracing the steps of metastases, cancer's menacing ballet. Science. 2003;299:1002.
[5] Houston SJ, Rubens RD. The systemic treatment of bone metastases. Clin. Orthop. 1995;312:95.
[6] Pritchard KI. The best use of adjuvant endocrine treatments. Breast. 2003;12:497.
[7] Lin A, Ray ME. Targeted and systemic radiotherapy in the treatment of bone metastasis. Cancer Metastasis Rev. 2006;25:669.
[8] Sim FH, et al. Orthopaedic management using new devices and prostheses. Clin. Orthop. 1995;312:160.
[9] Torchilin VP. Passive and active drug targeting: drug delivery to tumours as an example. Handb. Exp. Pharmacol. 2010;(197):3-53.
[10] Hirsjärvi S, Passirani C, Benoit JP. Passive and active tumour targeting with nanocarriers. Curr. Drug Discov. Technol. 2011 (in press)
[11] Hirabayashi H, Fujisaki J. Bone-specific drug delivery systems: approaches via chemical modification of bone-seeking agents. Clin. Pharmacokinet. 2003;42:1319.

[12] Wang D, Miller SC, Kopeckova P, Kopecek J. Bone-targeting macromolecular therapeutics. Adv. Drug Del. Rev. 2005;57:1049-1076.
[13] Masarachia P, et al. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. Bone. 1996;19:281.
[14] Coleman RE. Metastatic bone disease: clinical features, pathophysiology, and treatment strategies. Cancer Treat. Rev. 2001;27:165.
[15] Clezardin P, Ebetino FH, Fournier PGJ. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res. 2005;65:4971.
[16] Hashimoto K, Morishige K, Sawada K, Tahara M, Kawagishi R, Ikebuchi Y. et al. Alendronate inhibits intraperitoneal dissemination in in vivo ovarian cancer model. Cancer Res. 2005;65:540.
[17] Giraudo E, Inoue M, HanahanD. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J. Clin. Invest. 2004;114:623.
[18] Fujisaki J, Tokunaga Y, Takahashi T, Hirose T, Shimojo F, Kagayama A, et al. Osteotropic drug delivery system (ODDS) based on bisphosphonic prodrug. I: synthesis and in vivo characterization of osteotropic carboxyfluorescein.. J. Drug Target. 1995;3:273-282.
[19] Hirabayashi H, Sawamoto T, Fujisaki J, Tokunaga Y, Kimura S, Hata T. Relationship between physicochemical and osteotropic properties of bisphosphonic derivatives: rational design for osteotropic drug delivery system (ODDS). Pharm. Res. 2001;18:646-651.
[20] Niemi R, Vepsalainen J, Taipale H, Jairvinen T. Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel acyloxyalkyl esters of clodronic acid. J. Med. Chem. 1999;2:5053-5058
[21] Ezra A, Hoffman A, Breuer E, Alferiev S, Monkkonen, El Hanany- Rozen N, et al. A peptide prodrug approach for improving bisphosphonate oral absorption. J. Med. Chem. 2000;43:3641-3652
[22] Wright JE, Gittens SA, Bansal G, Kitov PI, Sindrey D, Kucharski C, et al. A comparison of mineral affinity of bisphosphonate-protein conjugates constructed with disulfide and thioether linkages. Biomaterials. 2006; 27(5):769-784.
[23] Ogawa K, Mukai T, Inoue Y, Ono M, Saji H. Development of a novel 99mTc-chelate conjugated bisphosphonate with high affinity for bone as a bone scintigraphic agent. J. Nucl. Med. 2006;47:2042-2047.
[24] Uludag H, Yang J, Targeting systemically administered proteins to bone by bisphosphonate conjugation. Biotechnol. Prog. 2002;18:604-611.
[25] Wang D, Miller S, Sima M, Kopeckova P, Kopecek J. Synthesis and evaluation of water-soluble polymeric bone-targeted drug delivery systems. Bioconj. Chem. 2003;14:853-859.
[26] Hengst V, Oussoren C, Kissel T, Storm G. Bone targeting potential of bisphosphonate-targeted liposomes. Preparation, characterization and hydroxyapatite binding in vitro. Int. J. Pharm. 2007;331:224-227.
[27] Miller K, Erez R, Segal E, Shabat D, Satchi-Fainaro R. Targeting bone metastases with a bispecific anticancer and antiangiogenic polymer-alendronate-taxane conjugate. Angew Chem. Int. Ed. Engl. 2009;48:2949-2954.

[28] Bala I, Hariharan S, Kumar MN. PLGA nanoparticles in drug delivery: the state of the art. Crit. Rev. Ther. Drug Carrier Syst. 2004;21:387-422.
[29] http://www.drugs.com/pro/alendronate.html; revision May, 2011 (last visit: July 2011).
[30] De Ruiter J, Clark R. Bisphosphonates: calcium antiresorptive agents. http://www.duc.auburn.edu/~deruija/endo_bisphos.pdf; revision Spring 2002 (last visit: July 2011).
[31] Pignatello R, Cenni E, Miceli D, Fotia C, Salerno M, Granchi D, et al. A novel biomaterial for osteotropic drug nanocarriers. Synthesis and biocompatibility evaluation of a PLGA-alendronate conjugate. Nanomed. 2009;4:161-175.
[32] Tosi G, Rivasi F, Gandolfi F, Costantino L, Vandelli MA, Forni F. Conjugated poly(D,L-lactide-co-glycolide) for the preparation of in vivo detectable nanoparticles. Biomaterials 2005;26:4189-4195.
[33] Cenni E, Granchi D, Avnet S, Fotia C, Salerno M, Micieli D, et al. Biocompatibility of poly(D,L-lactide-co-glycolide) nanoparticles conjugated with alendronate. Biomaterials. 2008;29:1400-1411.
[34] Choi SW, Kim JH. Design of surface-modified poly(D,L-lactide-co-glycolide) nanoparticles for targeted drug delivery to bone. J. Control. Release. 2007;122:24-30.
[35] Salerno M, Fotia C, Avnet S, Cenni E, Granchi D, Castelli F, et al. Effects of bone-targeted nanoparticles on bone metastasis. Calcified Tissue Int. 2008;82(Suppl. 1):S91.
[36] Cenni E, Salerno M, Fotia C, Avnet S, Granchi D, Castelli F, et al. Osteotropic poly(D,L-lactide-co-glycolide)-alendronate nanoparticles for the treatment of bone cancer. Cancer Treat. Rev. 2008;34(Suppl. 1):S74.
[37] Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, et al. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements for a screening strategy. Particle Fibre Toxicol. 2005;2:8.
[38] Borm PJA, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, et al. The potential risk of nanomaterials: a review carried out for ECETOC. Particle Fibre Toxicol. 2006;3:11.
[39] Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. Br. J. Pharmacol. 2005;146:882.
[40] Salvador-Morales C, Flahaut E, Sim E, Sloan J, Green MLH, Sim RB. Complement activation and protein adsorption by carbon nanotubes. Mol. Immunol.2006;43:193.
[41] Cenni E, Avnet S, Granchi D, Fotia C, Salerno M, Micieli D, et al. The effect of poly(D,L-lactide-co-glycolide) nanoparticles conjugated with alendronate on human osteoclast precursors. J. Mat. Sci. Polym. Med. 2011 (in press)
[42] http://www.drugs.com/pro/doxil.html (last visit: July 2011).
[43] Andreopoulou E, Gaiotti D, Kim E, Downey A, Mirchandani D, Hamilton A, et al. Pegylated liposomal doxorubicin HCL (PLD; Caelyx/Doxil®): Experience with long-term maintenance in responding patients with recurrent epithelial ovarian cancer. Ann. Oncol. 2007;18:716-721.
[44] Salerno M, Cenni E, Fotia C, Avnet S, Granchi D, Castelli F, et al. Bone-targeted doxorubicin-loaded nanoparticles as a tool for the treatment of skeletal metastases. Curr. Cancer Drug Targets. 2010;10:649-659.

[45] Calabresi P, Chabner A. In Goodman and Gilman's the pharmacological basis of therapeutics. Goodman Gilman A, Rall TW, Nies AS, Taylor P., eds. Chemotherapy of neoplastic diseases. The McGraw-Hill Companies: New York, 1991, pp. 1202-1263.

[46] Panyam J, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(D,L-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J. 2002;16:1217-1226.

[47] Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv. Drug. Deliv. Rev. 2007;59: 718-728.

[48] Clézardin P, Ebetino FH, Fournier PGJ. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res. 2005;65:4971-4974.

IntechOpen



Biomaterials Applications for Nanomedicine

Edited by Prof. Rosario Pignatello

ISBN 978-953-307-661-4
Hard cover, 458 pages
Publisher InTech
Published online 16, November, 2011
Published in print edition November, 2011

These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentialities of different synthetic and engineered biomaterials. Contributions were selected not based on a direct market or clinical interest, but on results coming from a very fundamental studies. This too will allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessary imposed by industrial or profit concerns. Biomaterial constructs and supramolecular assemblies have been studied, for example, as drug and protein carriers, tissue scaffolds, or to manage the interactions between artificial devices and the body. In this volume of the biomaterial series have been gathered in particular reviews and papers focusing on the application of new and known macromolecular compounds to nanotechnology and nanomedicine, along with their chemical and mechanical engineering aimed to fit specific biomedical purposes.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Rosario Pignatello (2011). PLGA-Alendronate Conjugate as a New Biomaterial to Produce Osteotropic Drug Nanocarriers, Biomaterials Applications for Nanomedicine, Prof. Rosario Pignatello (Ed.), ISBN: 978-953-307-661-4, InTech, Available from: http://www.intechopen.com/books/biomaterials-applications-for-nanomedicine/plga-alendronate-conjugate-as-a-new-biomaterial-to-produce-osteotropic-drug-nanocarriers

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen