

# Dexamethasone Dipropionate Loaded Nanoparticles of $\alpha$ -Elastin-g-PLGA for Potential Treatment of Restenosis

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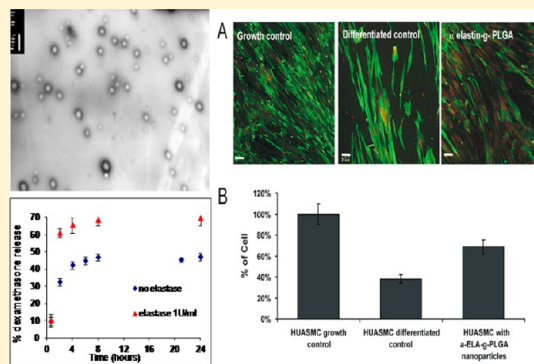
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**ABSTRACT:** A graft copolymer of  $\alpha$ -elastin with poly(lactic-co-glycolic acid) (PLGA) has been synthesized and successfully employed to produce nanoparticles. Exploiting the known biological activity of  $\alpha$ -elastin to promote the maintenance of smooth muscle cells (SMCs) contractile phenotype and the antiproliferative effect of glucocorticoids, the aim of this research was to produce drug-loaded nanoparticles suitable for potential treatment of restenosis. In particular, nanoparticles of  $\alpha$ -elastin-g-PLGA with a mean size of 200 nm have been produced and loaded with dexamethasone dipropionate (10% w/w), chosen as a model drug that inhibits proliferation of vascular SMCs. These nanoparticles are able to prolong the drug release and show a pronounced sensibility to elastase. Drug unloaded nanoparticles stimulate the differentiation of human umbilical artery smooth muscle cells (HUASMCs) toward the contractile phenotype as demonstrated by immunofluorescence, flow cytometric, and western blotting analyses. Finally, drug-loaded nanoparticles efficiently reduce viability of HUASMCs as evidenced by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay.

**KEYWORDS:**  $\alpha$ -elastin, poly(lactic-co-glycolic acid), graft copolymer, nanoparticles, restenosis, dexamethasone dipropionate



## INTRODUCTION

Vascular healing is a complex physiological process essential for maintaining vessel integrity and functionality. A dysfunction of this process can produce an intimal hyperplasia because of an abnormal migration and proliferation of vascular smooth muscle cells (VSMCs).<sup>1</sup> This hyperplasia can produce a phenomenon known as restenosis, that leads to the vessel obstruction where a tissue engineered vascular graft (TEVG) or a stent has been implanted. Thus, the inhibition of this hyperplasia is important to prolong patency of the implanted TEGV or stent functionality. At this aim, the specific target of therapeutic treatment is the key event of the hyperplasia, i.e., the proliferation of VSMCs. Indeed, VSMCs can assume both a proliferative and contractile phenotype;<sup>2</sup> in the first case, cells can rapidly proliferate and produce an extracellular matrix, while the change into a contractile phenotype would allow a better performance of TEGV or stent, thus reducing the risk of restenosis. Several drugs, growth factors, or extracellular matrix stimulating factors are potentially able to control the differentiation of VSMCs inhibiting the proliferative phenotype, and some of these agents are currently in therapeutic use.<sup>3,4</sup> In particular, several antiinflammatory drugs are employed to

reduce proliferation of VSMCs after a reconstructive surgery or they are loaded into stents to prevent restenosis.<sup>5,6</sup> However, the administration or loading of these drugs into stents without an appropriate carrier able to prolong their release makes such treatments often unsuccessful.<sup>7,8</sup>

Elastin is known to maintain a contractile phenotype of VSMCs,<sup>9,10</sup> inhibiting their migration and proliferation.<sup>1</sup> It has a morphogenetic role on differentiation of SMC, acting via a G-protein-coupled signaling pathway,<sup>1</sup> and it has demonstrated a reduction of restenosis after its administration in a pig model. Dexamethasone dipropionate inhibits proliferation of VSMCs,<sup>11</sup> blocking cell cycle progression late in the G1 phase, and it has already been employed to treat restenosis in stenting<sup>3</sup> both as free drug and loaded into polymeric nanoparticles.<sup>12</sup>

It is evident that, even if through a different mechanism, both elastin and dexamethasone are potentially employable to reduce

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restenosis and then if administered together they could have an interesting synergetic effect allowing to act both on SMC phenotype and directly on differentiated cells, inhibiting their proliferation. For this reason, in this work, we have prepared new nanoparticles based on an  $\alpha$ -elastin grafted to a biocompatible and biodegradable polyester, such as poly(lactic-co-glycolic acid) (PLGA), loaded with dexamethasone dipropionate. Physicochemical and biological properties of these nanoparticles have been evaluated, as well as their ability to prolong drug release.

## EXPERIMENTAL SECTION

**Materials and Methods.** Poly(lactic-co-glycolic) acid (PLGA) Resomer RG 502 H with an inherent viscosity of 0.19 dL/g,  $M_w$  3000 Da, was purchased by Boehringer-Ingelheim (Milan, Italy). Bis(4-nitrophenyl)carbonate (4-NPBC), 4-nitrophenyl chloroformate, elastin from bovine neck ligament, pancreatic elastase from porcine pancreas, pyridine, dexamethasone dipropionate (DEX), Dulbecco's phosphate buffered solution (DPBS), and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma-Aldrich (Milan, Italy). D-(+) trealose dihydrate and ethylenediamine (EDA) were purchased from Fluka (Milan, Italy).

FT-IR spectra were performed by using a Perkin-Elmer 1720 spectrophotometer in the range 4000–400  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR spectra were obtained with a Bruker AC-300 instrument. High performance liquid chromatography (HPLC) analysis has been performed by using an Agilent 1100 series with an Agilent injector 7725i and a UV detector Agilent 1100 equipped with a column Symmetry (Waters, C18, 5  $\mu\text{m}$  150  $\times$  4.6 mm), employing as an eluent acetate buffer 1 mM pH 4.8/acetonitrile in a ratio 25/75 v/v, with a flow of 0.8 mL/min and a  $\lambda$  value of 243 nm. Size exclusion chromatography (SEC) analysis was performed using a multidetector SEC system equipped with a Water 600 pump and a Water 410 Refractive Index Detector with Phenogel column employing as an eluent LiCl 0.05 M in DMF mixed with DMSO in a ratio 9:1 and a flow of 0.6 mL/min. Dynamic light scattering measurements were performed by using a Zetasizer Nano ZS instrument (Malvern Instrument, Germany). Transmission electron microscopy (TEM) images were recorded by using a JEOL JEM-2100 instrument.

**$\alpha$ -Elastin Extraction from Bovine Elastin.** The extraction of  $\alpha$ -elastin from elastin of bovine neck was performed following the procedure of Partridge et al.<sup>13</sup> Briefly, 1 g of bovine elastin was suspended in 0.25 M oxalic acid solution and hydrolyzed by sequential cycles of heating for 1 h in a water boiling bath. The supernatant was collected by centrifugation, and the precipitated solid was treated until complete hydrolysis. The collected soluble fractions were dialyzed against water using a dialysis membrane tube of 3500 Da and then freeze-dried. The obtained powder was dissolved in acetic acid solution 0.01 M containing NaCl 0.1 M (1 g of hydrolyzed elastin in 10 mL of acidic solution) and heated at 50 °C for 15 min to obtain the  $\alpha$ -elastin coacervation. The coacervate was collected by centrifugation. The procedure was repeated until complete precipitation of  $\alpha$ -elastin. The  $\alpha$ -elastin was finally dissolved in acetic acid solution 0.1 M and dialyzed against acetic acid solution using a dialysis membrane of 3500 Da at 4 °C for 1 day. Finally, the solution was freeze-dried and the  $\alpha$ -elastin was stored as a powder.

**Synthesis of PLGA-Ethylenediamine (PLGA-EDA) Derivative.** The synthesis of the PLGA-EDA derivative was performed similarly to the functionalization of polycaprolac-

tone, as described by Choi and colleagues,<sup>14</sup> with some modifications. In particular, pyridine and 4-nitrophenyl chloroformate have been added to a solution of PLGA in dichloromethane 10% w/v in a molar excess of 1.2 and 3, respectively. After 3 h at room temperature, the PLGA-nitrophenyl derivative was precipitated into an excess of diethyl ether, washed with ethanol, and dried under a vacuum (yield 100% w/w). The  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ ) showed the following peaks:  $\delta$  1.59 (2d, 3H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  of PLGA),  $\delta$  4.86 (s, 2H,  $-\text{CO}-\text{CH}_2-\text{O}-$  of PLGA),  $\delta$  5.22 (2d, 1H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  of PLGA),  $\delta$  8.03– $\delta$  8.87 (m, 4H, *p*-nitrophenyl moiety).

Ethylenediamine (EDA) was added to the solution of PLGA-nitrophenyl derivative (4% w/v) in dichloromethane, in a molar ratio EDA/PLGA-nitrophenyl derivative equal to 2. After 3 h at room temperature, the PLGA-EDA derivative was purified by precipitation into diethyl ether; then, the product was dissolved in acetone and precipitated into few volumes of NaCl saturated solution (100  $\mu\text{L}$  per gram of product). Finally, the product was washed with a mixture ethanol/water 8:2 and then with ethanol. The purification of the PLGA-EDA derivative was confirmed by HPLC analysis. The  $^1\text{H}$  NMR spectrum (in  $\text{CDCl}_3$ ) of PLGA-EDA derivative showed peaks at  $\delta$  1.59 (2d, 3H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  of PLGA),  $\delta$  2.2 (m, 2 H,  $-\text{CO}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}_2$  of EDA),  $\delta$  4.85 (s, 2H,  $-\text{CO}-\text{CH}_2-\text{O}-$  of PLGA), and  $\delta$  5.21 (2d, 1H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  of PLGA).

**Synthesis of  $\alpha$ -Elastin-g-PLGA Copolymers.** Three different weight ratios of PLGA-EDA/ $\alpha$ -elastin were employed equal to 2, 4, and 6 (samples a, b, and c, respectively), and a two-step reaction was performed in the following way.

Step 1:  $\alpha$ -elastin (3.3% w/v in anhydrous DMSO) was activated by employing 4-NPBC (weight ratio  $\alpha$ -elastin/4-NPBC equal to 6) for 3 h at 40 °C.<sup>15</sup> Step 2: the solution of activated  $\alpha$ -elastin was added to PLGA-EDA solution (40% w/v in anhydrous DMSO) in an amount corresponding to the chosen weight ratio reported above. The reaction solution was maintained at 40 °C for 24 h. Then, it was precipitated in ethanol and the product was washed with acetone. The  $\alpha$ -elastin-g-PLGA was obtained with a yield of 80% w/w. Purification from the unreacted PLGA-EDA was confirmed by SEC analysis.

The FT-IR spectrum (in KBr) showed the main following peaks: a broad band centered at 3450  $\text{cm}^{-1}$  ( $\nu_{\text{as}}$  OH +  $\nu_{\text{as}}$  NH of elastin), shoulder at 1780  $\text{cm}^{-1}$  ( $\nu_{\text{as}}$  COO of PLGA), 1655 and 1533  $\text{cm}^{-1}$  (amides of  $\alpha$ -elastin). The  $^1\text{H}$  NMR spectrum (in  $\text{DMF}-d_6$ ) showed the following peaks:  $\delta$  0.77 and 1.22 (s, 9H,  $-\text{NH}-\text{CH}(\text{CH}_3)\text{COO}-$  of alanine and  $-\text{NH}-\text{CH}(\text{CH}(\text{CH}_3)_2)-\text{COO}-$  of valine of  $\alpha$ -elastin, respectively),  $\delta$  1.59 (2d, 3H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  PLGA),  $\delta$  4.9 (s, 2H,  $-\text{CO}-\text{CH}_2-\text{O}-$  PLGA), and  $\delta$  5.25 (2d, 1H,  $-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  PLGA). The amount of PLGA grafted to  $\alpha$ -elastin was calculated comparing the area of the peaks attributable to protons of  $-\text{CO}-\text{CH}_2-\text{O}-$  and  $\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$  of PLGA ( $\delta$  4.9 and 5.25, respectively) with the area of peaks attributable to methyl groups of valine and alanine residues of  $\alpha$ -elastin.

In addition, to evaluate the activation efficiency of  $\alpha$ -elastin with 4-NPBC in the first step of the reaction, the 4-NPBC activated  $\alpha$ -elastin was precipitated in acetone and washed several times with the same solvent. The molar amount of 4-NPBC in  $\alpha$ -elastin was determined by colorimetric assay after treatment with NaOH 0.1 N. In particular, 5 mg of activated  $\alpha$ -

elastin was dissolved in NaOH 0.1 N and the absorbance was read at 405 nm. A calibration curve of 4-NPBC in NaOH 0.1 N was used for the quantitative determination of 4-NPBC released from activated  $\alpha$ -elastin. The molar percentage of  $\alpha$ -elastin activated by 4-NPBC was determined considering a mean molecular weight of 105 Da for aminoacids in  $\alpha$ -elastin as reported elsewhere.<sup>16</sup>

**$\alpha$ -Elastin-g-PLGA Nanoparticles: Preparation, Characterization, and in Vitro Drug Release Studies.**  $\alpha$ -Elastin-g-PLGA copolymer (sample a) was dispersed in DMSO with a concentration ranging from 2.5 to 15 mg/mL. To prepare drug-loaded nanoparticles, dexamethasone dipropionate was added in an amount corresponding to 0.5-fold the weight of copolymer. Dispersions were added drop by drop with a microsyringe into an excess of bidistilled water (ratio DMSO solution:bidistilled water equal to 1:15) under stirring at 9500 rpm by using a Ultraturax apparatus. The obtained dispersion was dialyzed against water for 3 days by using a Spectrapor dialysis tube (12000–14000 Da). Finally, a dialyzed dispersion was filtered through a 5  $\mu$ m cellulose acetate filter and freeze-dried. Size and zeta potential values of empty and drug-loaded nanoparticles were evaluated with a Zetasizer Nano ZS instrument in different media such as bidistilled water, DPBS pH 7.4, and DMEM. The size and shape of nanoparticles were also evaluated by TEM analysis. In particular, samples were prepared by dispersing  $\alpha$ -elastin-g-PLGA nanoparticles in bidistilled water (1 mg/mL) in a copper grid and drying at room temperature.

The amount of dexamethasone dipropionate loaded into nanoparticles was evaluated by HPLC analysis. In particular, 0.1 mL of a dispersion of drug-loaded nanoparticles in DMSO (10 mg/mL) was diluted with acetonitrile (1 mL) and then the obtained dispersion was filtered (0.45  $\mu$ m) before HPLC analysis. To evaluate the release of dexamethasone dipropionate, drug-loaded  $\alpha$ -elastin-g-PLGA nanoparticles were dispersed in DPBS pH 7.4 (1 mL) with or without the presence of elastase (1 U/mL) and sealed into a dialysis bag (14 000 Da) against 9 mL of DPBS pH 7.4, under stirring and at 37 °C. At scheduled times, 1 mL of external medium was collected and used for drug quantification by HPLC and then replaced by fresh DPBS pH 7.4. After 1 and 10 days, nanoparticle dispersion was recovered from the dialysis bag and freeze-dried to determine the amount of dexamethasone dipropionate remaining in the nanoparticles. Each experiment was performed in triplicate.

**In Vitro Culture of Human Umbilical Artery Smooth Muscle Cells.** Human umbilical artery smooth muscle cells (HUASMCs) were purchased from European Collection of Cell Cultures (ECACC). Cells were cultured in a basal growth medium (Smooth Muscle Cell Growth Medium from Cell Application, Inc. Cat No 311K-500) supplemented with 3% growth factors, 10% fetal bovine serum (FBS; Euroclone Celbio), 1% antibiotic, and 1% glutamine (Euroclone Celbio). Cells were cultured in flasks (75 cm<sup>2</sup>; Corning), and when confluent, they were detached using trypsin-EDTA solution 1 $\times$  (Sigma) and seeded on a culture multiwell plate (Corning) or on a coverslip, to perform different analyses.

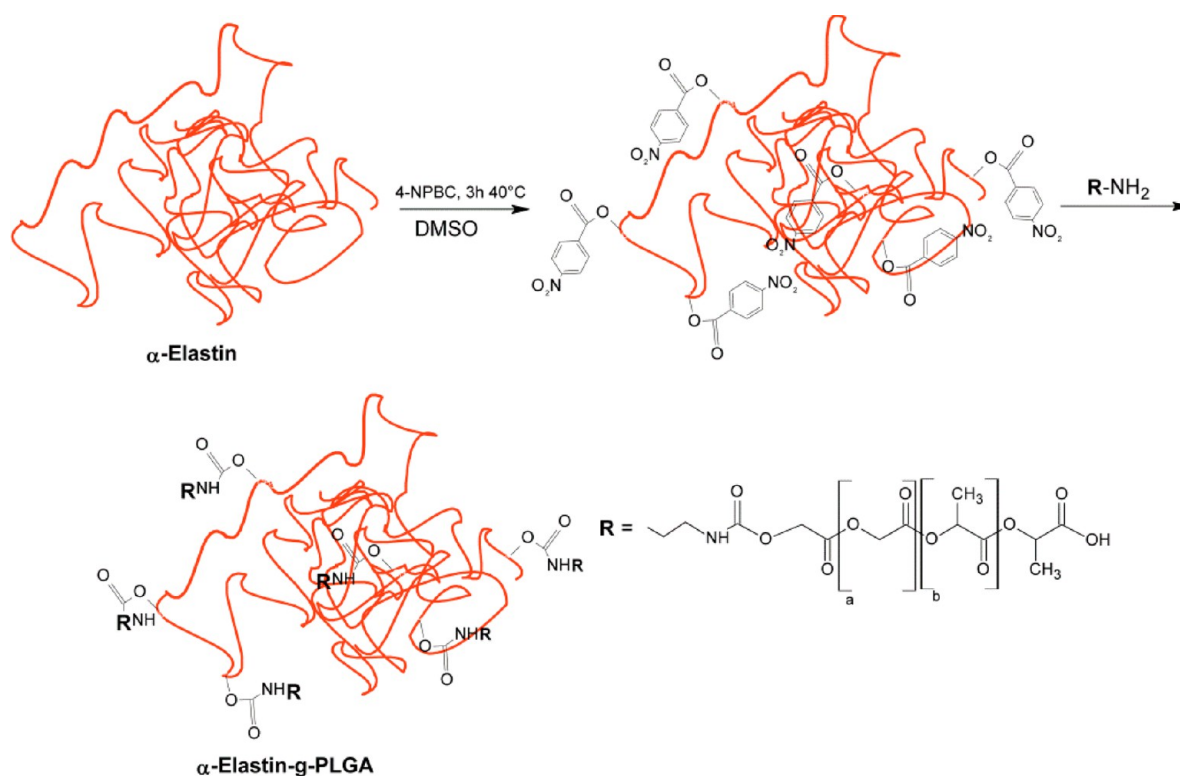
**Morphological Analysis.** HUASMCs were seeded at low density (5000 cells/well) on a sterile coverslip in a culture 12 well-plate. After 24 h, cells were incubated in a basal growth medium at 37 °C alone (growth control) or in the presence of 30  $\mu$ g/mL  $\alpha$ -elastin-g-PLGA nanoparticles. As differentiated control, cells were incubated with a differentiating growth

medium (Smooth Muscle Cell Differentiation Medium from Euroclone Celbio). This medium is specifically designed to promote differentiation of smooth muscle cells in culture and to maintain their mature contractile phenotype. The culture medium was changed every 2 days, and a new dose of nanoparticles (30  $\mu$ g/mL) was added each time. After 4 days of incubation, cells were fixed on a coverslip using formaldehyde 3.7% for 15 min and permeabilized with Triton 0.1%; then, the actin cytoskeleton was stained with phalloidin-FITC (1:500) for 1 h at 37 °C. Cells were thoroughly washed in phosphate buffer saline, and their nuclei were stained with propidium iodide (1:1000) for 1 h at 37 °C.<sup>17</sup> Cell proliferation was evaluated by quantification of phalloidin-FITC actin fibers/field related to the mean cell area for each sample in the different culture conditions (10 random fields for each sample), using the Image J64 software. Data were expressed as arbitrary units (A.U.). Morphological analysis was performed by confocal microscopy observation (Olympus 1  $\times$  70 with Melles Griot laser system).

**Flow Cytofluorimetric Analysis.** HUASMCs were seeded at high density (50 000 cells/well) in a culture 6 well-plate, and after 24 h, they were incubated as previously described. After 4 days of incubation, cells were detached using Tripsin-EDTA solution 1 $\times$  and counted using the Thoma chamber, and then, 800 000 cells/sample were centrifuged at 1000 rpm for 10 min. HUASMCs were treated with 500  $\mu$ L of propidium iodide solution (50  $\mu$ g/mL) and incubated for 45 min under dark conditions. The cell cycle was analyzed by FACS (BD FACSDiva version 6.1.2). Each experiment was performed in triplicate.

**Western Blotting Analysis.** HUASMCs were seeded at high density (50 000 cells/well) in a culture 6 well-plate, and after 24 h, they were incubated as previously described. After 4 days of incubation, cells were mechanically detached and centrifuged at 1000 rpm for 10 min. The extracts were obtained treating the pelleted cells with 70  $\mu$ L of 1% Triton X-100 solution for 10 min at room temperature and then centrifuged at 10 000 rpm for 10 min. The amount of proteins in the cell extracts was quantified by the Bradford microassay method (Bio-Rad, Segrate, Milan, Italy), using bovine serum albumin (Sigma-Aldrich) as a standard. A 5  $\mu$ g portion of each extract was analyzed by SDS-PAGE and blotted on nitrocellulose. By Western blotting analysis, the amount of  $\alpha$  smooth muscle actin for each sample was evaluated and quantified by densitometric analysis with Image J64 software. Each experiment was performed in triplicate.

**Cell Viability Assay.** HUASMCs were seeded at high density (50 000 cells/well) on a sterile coverslip in a culture 24 well-plate. After 24 h, cells were incubated in a basal growth medium for 48 h at 37 °C alone (control) or in the presence of drug-loaded  $\alpha$ -elastin-g-PLGA nanoparticles dispersed in DMEM. As a comparison, cells were also incubated in a basal growth medium in the presence of free drug dissolved in DMSO. The concentration of free dexamethasone was 50  $\mu$ g/mL, and the amount of drug-loaded  $\alpha$ -elastin-g-PLGA nanoparticles was 450  $\mu$ g/mL (this value corresponds to a drug concentration of 50  $\mu$ g/mL). A blank of  $\alpha$ -elastin-g-PLGA nanoparticles without drug was also assayed by using a concentration of 450  $\mu$ g/mL. The viability of each sample was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Cell Counting Kit; Sigma). After 3 h, the absorbance of each sample was read at 490 nm in a



**Figure 1.** Schematic representation of activation of  $\alpha$ -elastin with 4-NPBC and grafting of PLGA-EDA to obtain  $\alpha$ -elastin-g-PLGA copolymer.

GloMax-Multi Detection System (Promega). Each experiment was performed in triplicate.

## RESULTS AND DISCUSSION

Despite a wide application of native elastin in regenerative medicine,<sup>18</sup> its use for the production of nanoparticles for drug

**Table 1.** Grafting Degree in PLGA ( $GD_{PLGA}$ , mol %) for  $\alpha$ -Elastin-g-PLGA Copolymers Obtained by Using Different Weight Ratios between PLGA-EDA and  $\alpha$ -Elastin (Mean Value  $\pm$  Standard Deviation,  $n = 3$ )

weight ratio PLGA-EDA/ $\alpha$ -elastin	$GD_{PLGA}$ (mol %)
2 (sample a)	1.28 $\pm$ 0.04
4 (sample b)	1.38 $\pm$ 0.02
6 (sample c)	1.32 $\pm$ 0.02

**Table 2.** Values of Drug Loading (% w/w) and Loading Efficacy (% w/w) in  $\alpha$ -Elastin-g-PLGA Nanoparticles Prepared by Using Different Concentrations of Sample a (Mean Value  $\pm$  Standard Deviation,  $n = 3$ )

concentration of $\alpha$ -elastin-g-PLGA (sample a) (mg/mL)	drug loading (% w/w)	loading efficacy (% w/w)
2.5	1.0 $\pm$ 0.2	3 $\pm$ 0.1
5	3.0 $\pm$ 0.3	9 $\pm$ 0.3
7.5	10.0 $\pm$ 0.2	30 $\pm$ 0.2
15	13.0 $\pm$ 0.1	39 $\pm$ 0.2

release is limited by its poor solubility in several media. The acidic hydrolysis of desmosine bridges in native elastin allows the production of  $\alpha$ -elastin soluble both in aqueous and organic media.<sup>19</sup> Recently,  $\alpha$ -elastin has been employed for the production of nanoparticles by high temperature coacervation and cross-linking through  $\gamma$  irradiation.<sup>20</sup> On the other hand, it

**Table 3.** Values of Mean Diameter (MD), Polydispersity Index (PDI), and Zeta Potential (Pz) of Unloaded and Drug-Loaded  $\alpha$ -Elastin-g-PLGA Nanoparticles in Bidistilled Water, DPBS pH 7.4 and DMEM pH 7.4

$\alpha$ -elastin-g-PLGA nanoparticles	external medium	MD (nm)	PDI	Pz
drug unloaded	bidistilled water	198.8	0.356	-20.3
	DPBS pH 7.4	204.6	0.264	-9.9
	DMEM pH 7.4	184.8	0.307	-9.2
drug loaded	bidistilled water	224.9	0.390	-22.8
	DPBS pH 7.4	220.7	0.322	-10.8
	DMEM pH 7.4	221.0	0.382	-9.5

is well-known that nanocarriers are useful to improve bioavailability of poorly water-soluble drugs;<sup>21</sup> therefore, the grafting of a hydrophobic moiety on the  $\alpha$ -elastin chain could be exploited to produce appropriate nanoparticles able to entrap lipophilic drugs, such as dexamethasone dipropionate. For this reason, in this work, PLGA with a  $M_w$  value of about 3000 Da has been chosen as a hydrophobic moiety and grafted to  $\alpha$ -elastin through a simple and reproducible chemistry, even if  $\alpha$ -elastin has few reactive groups (more than 80% of aminoacids are alanin, valine, proline, and glycine).<sup>16</sup>

4-NPBC has been chosen as an activator of hydroxyl groups in  $\alpha$ -elastin (see Figure 1), and about 1.8 mol % of total aminoacids have been activated as evaluated by UV analysis (see the Experimental Section). This amount corresponds to about 50 mol % of hydroxyl groups containing aminoacids. At the same time, the reaction between PLGA activated by 4-nitrophenyl chloroformate and ethylenediamine (EDA) has been performed to obtain PLGA-EDA derivative. Then, the reaction between PLGA-EDA and the activated  $\alpha$ -elastin has been performed in anhydrous DMSO, by using three different

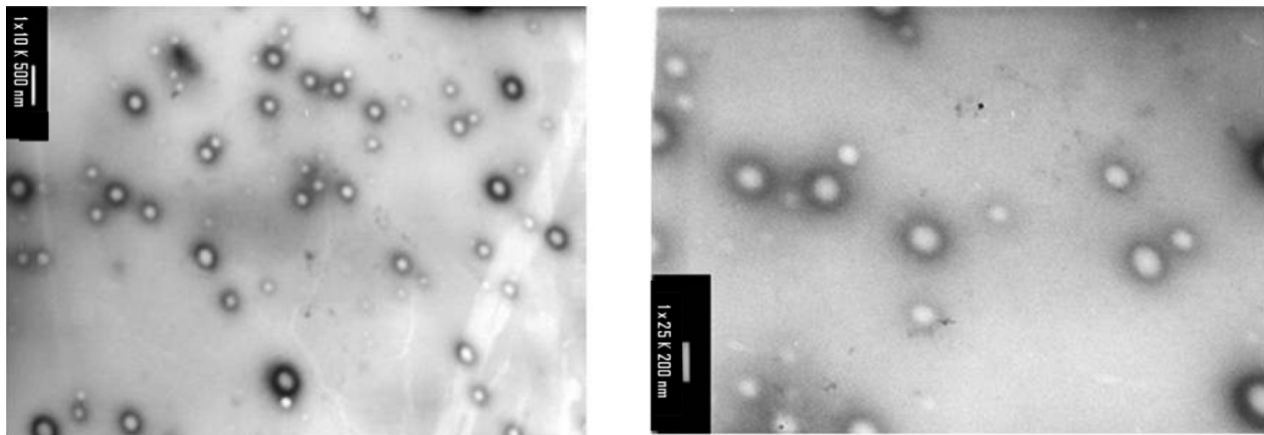


Figure 2. TEM pictures of drug unloaded  $\alpha$ -elastin-g-PLGA nanoparticles obtained at 10k (left) and 25k (right).

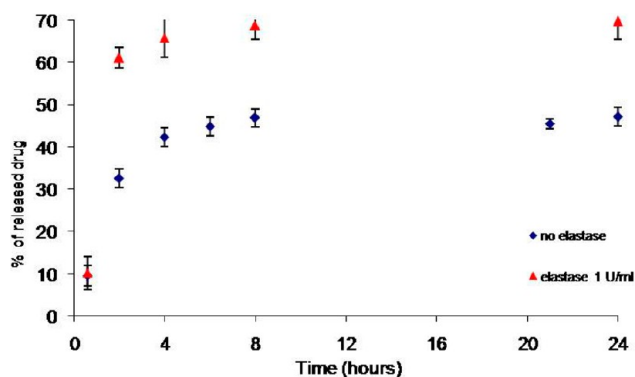


Figure 3. In vitro release profiles of dexamethasone dipropionate from  $\alpha$ -elastin-g-PLGA nanoparticles in DPBS pH 7.4 at 37 °C. Experiments were performed in the absence (◆) or in the presence (▲) of elastase (1 U/mL).

weight ratios of PLGA-EDA/ $\alpha$ -elastin (2, 4, or 6) in order to synthesize the corresponding  $\alpha$ -elastin-g-PLGA copolymer (sample a, b, or c) (see Figure 1).

$^1\text{H}$  NMR and FTIR analyses performed on each  $\alpha$ -elastin-g-PLGA copolymer have confirmed the success of the grafting procedure. Table 1 reports the amount of PLGA grafted to  $\alpha$ -elastin, calculated by  $^1\text{H}$  NMR analysis for all copolymers (see the Experimental Section).

It is evident that the grafting degree in PLGA does not change significantly by increasing the weight ratio of PLGA-EDA/ $\alpha$ -elastin, and a mean value equal to about 1.3 mol % has been obtained. This amount corresponds to the functionalization of about 40% of total free hydroxyl groups in  $\alpha$ -elastin, and this means that the 80% of aminoacids activated with 4-NPBC (that are 1.8 mol % as calculated by colorimetric assay) efficiently reacted with PLGA-EDA, thus confirming the success of the grafting reaction of PLGA-EDA on the  $\alpha$ -elastin chain.

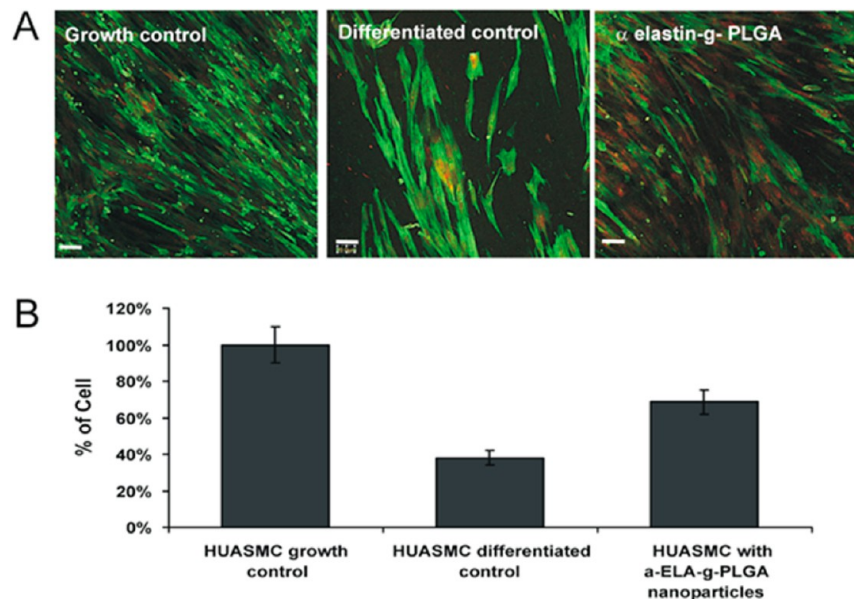
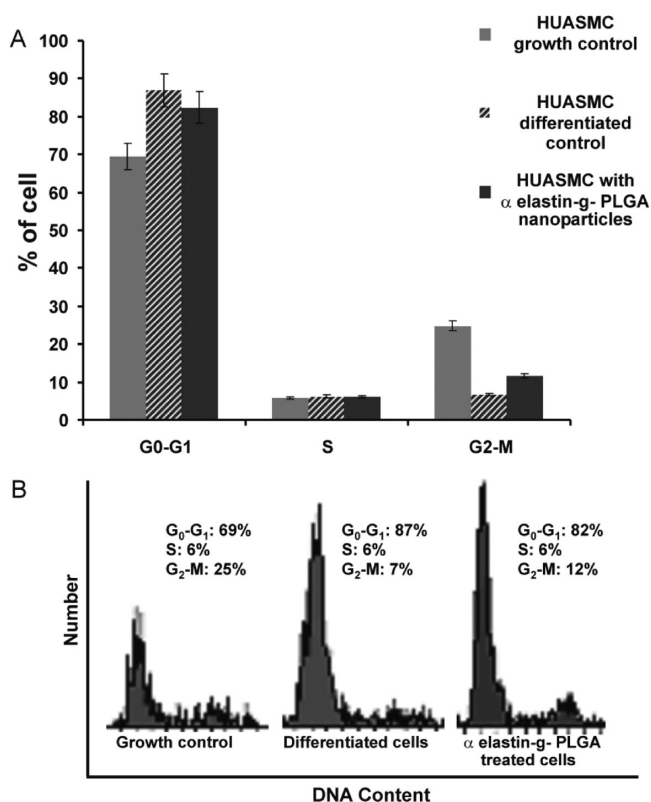
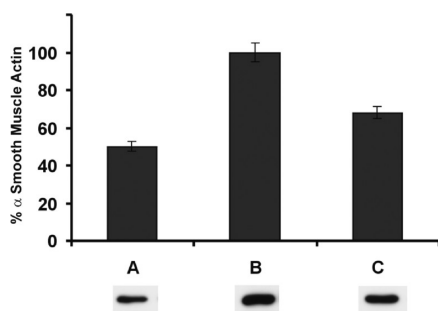


Figure 4. (A) Morphological analysis: HUASMCs incubated in a basal growth medium alone (growth control) or in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles, without drug. Cells incubated with differentiating growth medium have been used as a differentiated control. Cells were stained with phalloidin-FITC (green) and propidium iodide (red) and observed by a confocal microscope. (B) % of cells (as amount of red fluorescent nuclei) for each culture condition. The analysis was carried out by Image J64 software.



**Figure 5.** Flow cytometric profile of HUASMCs incubated in a basal growth medium alone (growth control) or in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles, without drug. Cells incubated with differentiating growth medium have been used as a differentiated control. The histograms report the % of cells in each phase of the cell cycle.



**Figure 6.** Western blotting analysis for  $\alpha$  SM actin expression from HUASMCs incubated in a basal growth medium alone (growth control, A) or in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles, without drug (C). Cells incubated with differentiating growth medium have been used as a differentiated control (B). The histograms report the densitometric evaluation of each line, determined by Image J64 software. The values were expressed as % of  $\alpha$  SM actin for each sample.

All  $\alpha$ -elastin-g-PLGA copolymers were not soluble in water but very soluble in DMSO; however, considering the similar grafting degree in PLGA, only one copolymer (sample a) has been chosen for the production of nanoparticles. In particular, as reported in the Experimental Section,  $\alpha$ -elastin-g-PLGA nanoparticles have been prepared by the nanoprecipitation technique using a copolymer (sample a) concentration ranging from 2.5 to 15 mg/mL in DMSO and distilled water has been used as a precipitating medium. Drug-loaded nanoparticles have been prepared by dissolving dexamethasone dipropionate in

DMSO in an amount corresponding to 0.5-fold the weight of copolymer.

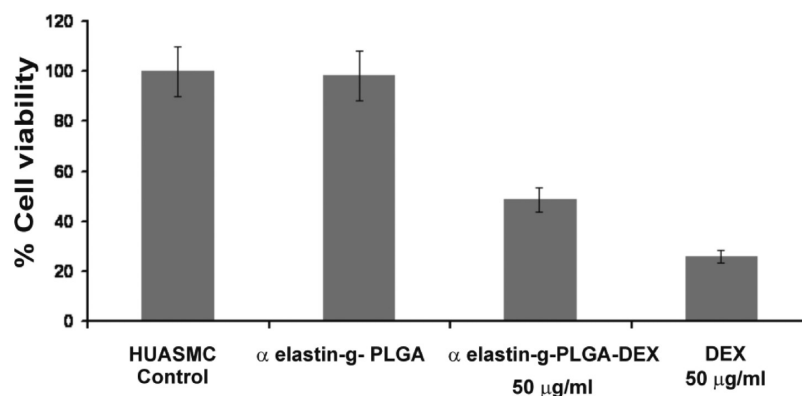
As shown in Table 2, both drug loading and loading efficacy increase with copolymer concentration, reaching a value of 13 and 39% w/w, respectively, for a concentration of 15 mg/mL. However, at this concentration, drug-loaded nanoparticles were not dispersible in aqueous medium; on the contrary, nanoparticles obtained with a copolymer concentration of 7.5 mg/mL give a fine aqueous dispersion. For this reason, nanoparticles having a drug loading of 10% w/w and a loading efficacy of 30% w/w have been used for further characterization.

Table 3 reports the values of mean diameter and zeta potential of these nanoparticles determined in different media by dynamic light scattering measurements.

For each sample, no significant difference has been found by changing the external medium. The presence of drug causes an increase of about 10% in nanoparticle diameter, but it does not affect the value of the zeta potential, thus indicating that dexamethasone dipropionate is above all entrapped inside the nanoparticle and not on the surface. TEM analysis has confirmed dynamic light scattering data, showing nanoparticles of about 200 nm with a narrow size distribution. Figure 2 reports, as an example, TEM images of drug unloaded nanoparticles.

The release profile reported in Figure 3 shows that, in the absence of enzyme, a plateau is obtained after 8 h and only about 40% of drug is released. The presence of elastase (1 U/mL) causes a pronounced increase in the amount of released drug that, after 4 h, is about 70% with respect to the total amount. This result demonstrates that  $\alpha$ -elastin even if grafted to PLGA maintains its susceptibility toward elastase that is able to hydrolyze its chains, thus allowing a faster drug release. However, after 24 h, a significant amount of drug remains still entrapped into the nanoparticles, both in the absence and in the presence of elastase. Unfortunately, after 24 h, dexamethasone dipropionate released in DPBS pH 7.4 undergoes a significant hydrolysis, giving rise to dexamethasone monopropionate and dexamethasone, thus making the analysis of release profile for longer times difficult. However, in order to evaluate if  $\alpha$ -elastin-g-PLGA nanoparticles are able to prolong the drug release, after 10 days, nanoparticle dispersion was recovered from the dialysis bag and freeze-dried to determine the amount of dexamethasone dipropionate remaining in the nanoparticles. This amount resulted in being the 10% w/w for nanoparticles treated with elastase and 25% w/w for nanoparticles in the absence of elastase. The obtained result suggests that  $\alpha$ -elastin-g-PLGA nanoparticles are able to release in a prolonged way (for more than 10 days) a poorly soluble drug, such as dexamethasone dipropionate.

As reported in the Introduction, both elastin and dexamethasone have an inhibitory effect on SMC proliferation, even if with a different mechanism. Therefore, with the aim of differentiating between drug-loaded nanoparticles and non-drug-loaded nanoparticles, different biological assays have been performed, i.e., (1) morphological, flow cytofluorimetric, and Western blotting analyses to evaluate the role of  $\alpha$ -elastin in  $\alpha$ -elastin-g-PLGA nanoparticles in the absence of drug and (2) a MTS assay to evaluate the antiproliferative effect of dexamethasone dipropionate loaded into  $\alpha$ -elastin-g-PLGA nanoparticles in comparison with empty  $\alpha$ -elastin-g-PLGA nanoparticles and free dexamethasone dipropionate.



**Figure 7.** Viability of HUASMCs determined by MTS assay after their incubation with basal growth medium alone (control) or in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles without drug (blank), drug-loaded  $\alpha$ -elastin-g-PLGA nanoparticles (450  $\mu$ g/mL), or free drug (DEX, 50  $\mu$ g/mL). The amount of drug-loaded  $\alpha$ -elastin-g-PLGA nanoparticles (450  $\mu$ g/mL) corresponds to a DEX concentration of 50  $\mu$ g/mL.

In particular, by morphological analysis, the potential of  $\alpha$ -elastin-g-PLGA nanoparticles to stimulate the differentiation toward the contractile phenotype of HUASMCs during the *in vitro* culture has been evaluated. Cells have been incubated with basal growth medium alone (growth control) or in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles. Cells incubated with differentiating growth medium have been used as a differentiated control (see the Experimental Section). In agreement with data reported in the literature, the morphological analysis (Figure 4A) evidenced that cells treated with  $\alpha$ -elastin-g-PLGA nanoparticles show in their cytoplasm a highly organized network of actin stress fibers, a hallmark of mature contractile vascular smooth muscle cells, like the differentiated control.

An image analysis has been performed using Image J64 software to evaluate the number of cells for each culture condition. In particular, the % of red fluorescent nuclei for each sample was quantified and reported in Figure 4B. It is evident that the % of HUASMCs decreases with the following order: control cells (considered as 100% of proliferation) > cells treated with nanoparticles (69%) > differentiated control (38%). This result suggests that proliferation of cells treated with  $\alpha$ -elastin-g-PLGA nanoparticles (without drug) decreases due to the gradual differentiation toward the contractile phenotype.

To confirm these data, flow cytometric analysis has also been performed. As it is possible to observe in Figure 5, for HUASMCs treated with  $\alpha$ -elastin-g-PLGA nanoparticles (without drug), like for differentiated control, the % of cells in phase G0–G1 is greater than growth control, thus confirming the differentiation toward a contractile phenotype due to the presence of  $\alpha$ -elastin-g-PLGA nanoparticles.

Moreover, by a Western blotting analysis, the amount of  $\alpha$  smooth muscle actin ( $\alpha$  SM actin) has been determined, with this protein being a specific marker of SMC differentiation.<sup>22</sup> As shown in Figure 6, the % of  $\alpha$  SM actin in cells treated both with  $\alpha$ -elastin-g-PLGA nanoparticles and with differentiating growth medium is higher than growth control, thus confirming again that the presence of  $\alpha$ -elastin-g-PLGA nanoparticles, without drug, promotes the differentiation toward the contractile phenotype.

However it is appropriate to relate the percentage of expression of  $\alpha$  SM actin with the mean area of the cells cultured under different conditions. Indeed, it is important to specify that, for sample A (growth control) of Figure 6, the  $\alpha$  SM actin expression (basal level) is due to a high number of

cells present in the control. On the contrary, the  $\alpha$  SM actin expression for sample C (HUASMCs treated with  $\alpha$ -elastin-g-PLGA nanoparticles) is due to a lower number of cells (with a greater mean area) having a differentiated contractile phenotype.

Finally, a MTS assay has been performed to evaluate the antiproliferative effect of dexamethasone dipropionate loaded into nanoparticles. HUASMCs incubated in basal growth medium were used as a control, whereas  $\alpha$ -elastin-g-PLGA nanoparticles without drug were used as a blank. Results have been compared with those obtained by using free dexamethasone dipropionate dissolved in DMSO.

As shown in Figure 7, viability of HUASMCs incubated for 48 h in the presence of  $\alpha$ -elastin-g-PLGA nanoparticles without drug (blank) is equal to that of cells treated with basal growth medium alone. On the contrary, drug-loaded nanoparticles cause a pronounced decrease in cell viability like free dexamethasone dipropionate. The obtained results demonstrate that, according to the different action mechanism of  $\alpha$ -elastin and dexamethasone, as mentioned above, MTS assay allows one to evaluate only the antiproliferative effect of drug entrapped into nanoparticles.

To sum up, biological assays differentiate between drug-loaded and non-drug-loaded nanoparticles, thus demonstrating the benefit due to the combination of  $\alpha$ -elastin-g-PLGA and dexamethasone dipropionate for a potential treatment of restenosis.

## CONCLUSIONS

A simple and reproducible grafting reaction has been described to obtain an  $\alpha$ -elastin-g-PLGA copolymer employed for the production of nanoparticles potentially useful to prevent restenosis. In particular, nanoparticles with a mean diameter of 200 nm have been produced and successfully employed to entrap dexamethasone dipropionate, a known drug that inhibits proliferation of vascular smooth muscle cells (VSMCs). Thanks to the activity of  $\alpha$ -elastin in regulating smooth muscle cell phenotype,  $\alpha$ -elastin-g-PLGA nanoparticles alone are able to stimulate the differentiation of human umbilical artery smooth muscle cells toward the contractile phenotype, as evidenced by different biological studies like immunofluorescence, flow cytometric, and western blotting analyses. Moreover, drug-loaded nanoparticles exhibit in aqueous medium a good antiproliferative effect comparable to that of free drug dissolved in organic medium. Therefore,  $\alpha$ -elastin-g-PLGA nanoparticles

show an intrinsic ability to promote the differentiation toward the contractile phenotype combined with the antiproliferative effect of dexamethasone dipropionate. In addition, release studies have shown that  $\alpha$ -elastin-g-PLGA nanoparticles act as a drug reservoir, thus allowing a prolonged release in aqueous medium without the use of organic solvent. The obtained results can be considered as positive preliminary data for a further design of innovative devices for restenosis treatment.

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### Notes

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