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Polysaccharide/polyaminoacid composite scaffolds for modified DNA release

G. Pitarresi[a,*], R. Calabrese[a], F.S. Palumbo[a], M. Licciardi[a], G. Giammona[a,b]

[a] Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, via Archirafi 32, 90123, Palermo, Italy
[b] IBF-CNR, Via Ugo La Malfa 153, 90143, Palermo, Italy

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ABSTRACT

In this work composite polymeric films or sponges, based on hyaluronic acid (HA) covalently crosslinked with α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE), have been prepared and characterized as local gene delivery systems. In particular, HA/PE scaffolds have been loaded with PE/DNA interpolyelectrolyte complexes, employing PE as a macromolecular crosslinker for HA and as a non-viral vector for DNA. In vitro studies showed that HA/PE films and sponges have high compatibility with human dermal fibroblasts and they give a sustained DNA release, whose trend can be easily tailored by varying the crosslinking ratio between HA and PE. Electrophoresis analysis and transfection studies on B16-F10 cells revealed that DNA is released as a complex with PE and it retains its bioactivity.
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1. Introduction

The difficulty in local administration of growth factors or other bioactive peptides for tissue repair and regeneration is due to well known problems linked to their formulation or to their rapid clearance. On the other hand, local gene transfer gives the opportunity for the intracellular introduction of therapeutic genes in the site of injury or in the damaged tissue, thus inducing the host cells to produce growth factors or cytokines to create a new functional tissue (Middaugh et al., 1998; Bonadio, 2000, 2002; Pannier and Shea, 2006; Yamamoto and Tabata, 2006).

An emergent approach in the field of tissue repair and regeneration consists in the production of gene activated matrices or GAMs, a new kind of advanced biomaterials based on the combination of tissue engineering and gene delivery approaches (Pannier and Shea, 2004; Bumerot et al., 2006; Storrie and Mooney, 2006). A GAM provides a scaffold that, once placed into a tissue lesion or on a wound bed, lets cells adhere, growth and proliferate and stimulates cells to produce bioactive peptides or proteins necessary for tissue regeneration (Wang et al., 1999; Scherer et al., 2002; Kim et al., 2003, 2005; Cohen-Sacks et al., 2004; Huang et al., 2005).

DNA can be loaded naked into a scaffold or as a reversible complex with a lipid or polycation carrier (De Schmedt et al., 2000; Jones et al., 2000; Luo et al., 2000; Cavallaro et al., 2006; Licciardi et al., 2006). In particular, polycations are able to condense, protect and package DNA to deliver it into cells and many polycations can also promote gene expression facilitating DNA release from endosomes into the cytoplasm by a proton buffer effect (Akinc et al., 2005; Park et al., 2006).

The preparation of biocompatible and biodegradable films, based on hyaluronic acid (HA) crosslinked with α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE), having suitable biological and physicochemical properties as scaffolds for tissue engineering, was previously reported (Pitarresi et al., 2008). In this study, HA–PE films or sponges containing DNA as a complex with the PE were prepared and characterized. The production of composite polysaccharide/polyaminoacid materials is one useful strategy to obtain systems with suitable physicochemical and biological properties: by varying the polysaccharide/polyaminoacid molar ratio, swelling degree, hydrolytic resistance, rate of degradation, bioadhesive behaviour and other key properties can be easily tailored in function of specific biological or pharmaceutical needs. Moreover, the combination of a natural macromolecule like HA with a synthetic polymer like PE, allows to exploit the typical biocompatibility of the first polymer and the advantageous properties of the latter, such as chemical versatility, easy processing and low cost production. In this work the advantages of the synthetic polyaminoacid PE are fully exploited, using it not only as a macromolecular crosslinker for HA, but also as a non-viral carrier for DNA, by means of polyplexes formation before incorporation into scaffolds. The choice of PE as DNA polymeric vector was dictated by the interesting biophysical and biological properties it showed in this role, such as the good DNA complexing ability, high biocompatibility and transfection ability (Cavallaro et al., 2007).

* Corresponding author. Tel.: +39 0916236154; fax: +39 0916236150.
E-mail address: giopitar@unipa.it (G. Pitarresi).

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The produced HA–PE scaffolds containing PE/DNA polyplex were characterized in vitro in order to evaluate their biocompatibility and ability to release DNA in physiological simulated fluid in the presence or in the absence of hyaluronidase. In order to evaluate the biological activity of DNA released from the produced scaffolds, in vitro cell transfection studies were also performed.

2. Materials and methods

All reagents were of analytical grade, unless otherwise stated. Ethylenediamine, bis(4-nitrophenyl)carbonate (4-NPBC), N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Fluka (Milano, Italy). Anhydrous N,N-dimethylformamide (DMF), deuterium oxide (D_2O, isotopic purity 99.9%), Dulbecco's phosphate buffered saline (DPBS), bovine testicular hyaluronidase (HAase, 1320 units/mg), minimum essential medium Eagle (MEM), Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA solution, amphotericin B solution, penicillin–streptomycin solution, fetal bovine serum (FBS), DNA from calf thymus (sodium salt), lambda HindIII DNA, were obtained from Sigma Aldrich (Milano, Italy). MTS/PES solution (*CellTiter 96® AQ_{uqeous} One solution Cell Proliferation Assay*) was purchased from Promega (Italy). Pico Green reagent was purchased from Invitrogen (Italy). Plasmid pCMVLuc *Photinus pyralis* luciferase under control of the cytomegalovirus (CMV) enhancer/promoter (Plank et al., 1992) was produced endotoxin-free by Elim Biopharmaceuticals (San Francisco, USA) or Aldevron (Fargo, ND, USA).

Hyaluronic acid (HA) sodium salt, M_w 1500 kDa was a generous gift from Sifi (Catania, Italy). HA with a low weight-average molecular weight, employed in our experiments, was prepared by acidic degradation, as reported by Shu et al. (2002). Briefly, 1% (w/v) solution of hyaluronic acid sodium salt was degraded in HCl solution (pH 0.5) at 37 °C, under orbital stirring (150 rpm), for 24 h. After this time, the pH was corrected to 7.0 and the solution subjected to a dialysis, by using Spectrapor Tubing with a molecular cut-off of 3500. After dialysis, the solution was freeze-dried and the weight-average molecular weight of HA was determined by size exclusion chromatography (SEC) analysis using a HPLC 515 pump equipped with a Universal column (particle size 5 μm) and a 410 differential refractometer as a concentration detector, all from Waters (USA). The molecular weight, evaluated by using 200 mM phosphate buffered solution (pH 6.5): MeOH 90:10 (v/v) as a mobile phase, 36 ± 0.1 °C and flow rate of 0.6 ml/min, was 222 kDa (M_w/M_n = 1.85) based on HA standards (range 100–800 kDa) from Hyalose (USA).

The α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to a procedure reported elsewhere (Giammona et al., 1987). Spectroscopic data (FT-IR and ^{1}H NMR) were in agreement with previous results (Mendichi et al., 2000). PHEA weight-average molecular weight was measured by SEC analysis by using a HPLC 515 pump equipped with a Universal a 410 differential refractometer as a concentration detector and two Ultrahydrogel columns (500 and 200 Å), all from Waters (USA), and a phosphate buffered solution at pH 8 as mobile phase at 37 °C with a flow rate of 0.6 ml/min and it was 41 kDa (M_w/M_n = 1.68) based on poly(ethylene oxide)/poly(ethylene glycol) (PEO/PEG, range 145–1.5 kDa) standards (Mendichi et al., 2000).

The α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE) was obtained from PHEA as previously described (Cavallaro et al., 2006), by means of derivatization with ethylenediamine (EDA) in anhydrous N,N-dimethylformamide (DMF), using bis(4-nithrophenyl)carbonate (4-NPBC) as an activator. Briefly: a solution of PHEA (0.25 g, 1.57 mmol of repeating units) in 3 ml of anhydrous DMF was added dropwise to a DMF solution of 4-NPBC, according to R_1 = 1, where R_1 = moles of 4-NPBC/moles of PHEA repeating units. The reaction was kept at 40 °C under continuous stirring for 4 h. Subsequently, the reaction mixture was added to EDA, according to R_2 = 5, where R_2 = moles of EDA/moles of PHEA repeating units, and the reaction was kept at 20 °C under continuous stirring for 4 h. After this time, the reaction mixture was precipitated into acetone and, after centrifugation for 10 min at 9800 rpm and 4 °C, the product was recovered and washed several times with acetone. The obtained solid residue was dissolved in twice-distilled water and purified by dialysis using Visking Dialysis Tubing with a molecular weight cut-off of 12,000–14,000. After dialysis, the solution was freeze-dried. The PE derivative was obtained with a yield of 90–93% (w/w), based on the starting PHEA. Spectroscopic data were in agreement with previous results (Cavallaro et al., 2006). The derivatization degree of PE derivative was calculated by ^{1}H NMR spectroscopic data and it resulted 55 ± 3 mol%. PE weight-average molecular weight, measured by SEC analysis with the same conditions already described for PHEA, was 35 kDa (M_w/M_n = 1.68), based on PEO/PEG standards (Cavallaro et al., 2006).

Centrifugations were performed with an International Equipment Company Centra MP4R equipped with an 854 rotor and temperature control. Scaffolds morphological analysis was performed using a scanning electron microscope (SEM) Leo stereoscan 420; each sample was made conductive by depositing a gold layer on its surface under vacuum; the scaffold pore size was determined by measuring random 25 pores from SEM image by image analysis software. DNA release studies were performed in a Benchtop 80 °C Incubator Orbital Shaker 420. For the Pico Green assay was used an FP-6500 Jasco spectrofluorimeter. Cell proliferation MTS assays were performed using a Thermo Labsystems Multiskan Ex 96-well microplate photometer. Cell transfection analyses were performed using a Promega Glomax 20/20 single auto injector luminometer.

2.1. Cell culture

Human dermal fibroblasts and murine melanoma cells B16-F10, purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Umbertini" (Italy), were cultured in MEM or DMEM, respectively, supplemented with 15% of FBS and 1% of penicillin–streptomycin and amphotericin B solution and maintained in a 5% CO_2 incubator with humidified atmosphere at 37 °C. When cells reached 80% confluence, they were detached using a trypsin/EDTA solution and they were then resuspended in complete fresh medium at the required density.

2.2. Polyplexes formation between α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE) and DNA

The formation of polyplexes between PE polycation and DNA was investigated in tris-acetate/EDTA (TAE) buffer pH 7.2. Briefly, polycation was dissolved in TAE buffer at room temperature (1.2 mg/ml) and calf thymus or pCMVLuc DNA solutions were prepared in the same medium at a 0.3 mg/ml concentration. Complexation was performed by mixing aliquots of DNA and polycation solutions resulting in a final polycation/DNA weight ratio equal to 4 and incubating the obtained solution for 1 h at 37 °C under gentle stirring. PE/DNA polyplexes thus obtained were extensively subjected to biophysical and biological characterization. In particular, gel electrophoresis retardation assay, dynamic light scattering and zeta potential measurements, toxicity and reporter gene assays, stability and DNAase protection assays, erythrocyte and haemolytic tests, and intracellular trafficking studies were performed as reported elsewhere (Cavallaro et al., 2007) and data

obtained were in agreement with our previous results (Cavallaro et al., 2007).

2.3. Preparation of hyaluronic acid (HA) and α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE) based scaffolds in the presence of PE/DNA polyplexes

Scaffolds shaped as films or sponges based on HA chemically crosslinked with PE and loaded with PE/DNA polyplexes, were produced as described below. Stock solutions of the starting polymers HA and PE in aqueous NaCl (0.9%, w/v) were prepared and the pH of the obtained solutions was regulated to 7.4 using NaOH/HCl 0.1 N. Glycerol (10%, w/v) was added to the film forming solutions. Subsequently, HA and PE stock solutions were mixed in adequate amount to obtain three different crosslinking ratio (indicated as X values), equal to 0.5, 1.0 or 1.5, being X = moles of amino groups of PE/moles of HA repeating units. Final HA concentration in each gel forming solution was 3% (w/v) for the films and 1.7% (w/v) for the sponges. After vigorous mixing, N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC) was added to the polymeric solutions, according with Y value equal to 0.7 and 1.0 for films and sponges, respectively, where Y = moles of activator (EDC or NHS)/moles of amino groups in PE. After a few minutes, the polyplexes PE/DNA solution, prepared just before use, was added to the polymer solutions resulting in a final DNA concentration equal to 0.02 mg/ml. After mixing, N-hydroxysuccinimide (NHS) was added to the polymeric solutions, according with Y value equal to 0.7 and 1.0 for films and sponges, respectively. After a few seconds, the gel forming solutions were poured into Petri's disks (diameter = 3.2 cm) and kept at room temperature and atmospheric pressure for 3 h.

The obtained hydrogels were purified and dried in a different way, depending on the final shape required. In particular, in order to produce sponges, the hydrogels obtained from the solution with a lower HA content (1.7%, w/v), were purified by washing with twice-distilled water (4 × 100 ml) and subsequent freeze-drying. Conversely, in order to obtain films, the solution with a higher HA content (3%, w/v) were purified by washing (4 × 100 ml) with glycerol aqueous solution (10%, w/v in twice-distilled water) and then they were dried at room temperature and atmospheric pressure.

The DNA loading efficiency of the obtained HA–PE based scaffolds was expressed as:

$$\text{DNA loading efficiency}\,(\%) = \frac{\text{mg DNA loaded into the scaffold}}{\text{mg DNA initially input}} \times 100$$

where amount of DNA loaded into the scaffolds was indirectly calculated by quantifying the DNA present in the washing media with spectrofluorimetric Pico of Green assay, performed according to a procedure already reported in literature (Labarca and Paigen, 1980), and by subtracting it to the initial amount of DNA input.

DNA loading efficiency % thus determined was 71.0 ± 0.9% and 73.2 ± 1.7% for sponges and films, respectively.

2.4. In vitro cell-compatibility studies

In order to evaluate the cell-compatibility of HA–PE films and sponges and their potential degradation products, MTS direct and indirect assays were performed, using human dermal fibroblasts as a model cell line.

For the direct cell-compatibility assay, sterile circular samples of HA–PE films and sponges (6 mm in diameter) unloaded or loaded with PE/DNA polyplexes, were placed into 96-well plates and immersed in 200 μl of complete MEM for 60 min. The medium was then removed from each well and replaced with 100 μl of cell suspension in complete MEM (100,000 cells/ml) and the plates were placed into the cell incubator (37 °C, humidified atmosphere, 5% CO_2) for 48 h. After this time, the culture medium was removed and the cells adhered to the scaffolds were detached by treating with trypsin/EDTA solution. Subsequently, the scaffolds were removed, 100 μl of fresh complete MEM and 20 μl of MTS/PES reagent solution were added to each well and the plates were placed into the cell incubator. After 2 h the absorbance at 492 nm was recorded for each sample. Since the absorbance at 492 nm is proportional to cell viability, the obtained results were finally reported as:

$$\text{Cell viability}\,(\%) = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

being A_{sample} = absorbance at 492 nm of the sample and A_{control} = absorbance at 492 nm of the positive control, using as positive control cells in complete MEM (100,000 cells/ml) incubated in the absence of the scaffolds. Each experiment was performed in triplicate.

For the indirect cell-compatibility assay, HA–PE films and sponges were incubated in MEM without FBS at 37 ± 0.1 °C, under orbital stirring at 120 rpm, for 12 or 24 days. After incubation, the medium was centrifuged for 30 min at 11,800 rpm and 4 °C, filtered and frozen up to the time of use (this medium is further indicated as "conditioned medium"). Human dermal fibroblasts were seeded in complete MEM (100,000 cells/ml) in a 96-well plate (0.1 ml/well), and placed into the cell incubator (37 °C, humidified atmosphere, 5% CO_2) for 24 h. Subsequently, the culture medium was replaced with the conditioned media, previously thawed and supplemented with 15% FBS. After 48 h of incubation, the medium was replaced with fresh complete MEM and MTS assay was performed as described above. Each experiment was performed in triplicate.

2.5. DNA release studies

Circular samples (6 mm in diameter) of HA–PE films (26 mg) and sponges (4 mg), containing, respectively 7.5 and 2 μg of PE/DNA polyplexes or naked DNA and having X = 1.5, 1.0 or 0.5, were immersed in 1.5 ml of pH 7.4 phosphate buffered solution (PBS) in the presence or in the absence of hyaluronidase (HAase, 10 U/ml) and they were incubated at 37 °C for 28 days. At established times, the aqueous medium was collected for Pico Green assay, electrophoretic analysis and transfection studies and replaced with fresh medium.

2.5.1. Pico green assay

The amount of released DNA was measured by means of the spectrofluorimetric Pico Green assay, performed according with a procedure already reported in literature (Labarca and Paigen, 1980). In brief, 1 ml of each DNA released sample was mixed 1:1 with the Pico Green reagent, previously 200-fold diluted with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5), and this solution was incubated for 5 min at room temperature protected from the light. After the incubation, the sample fluorescence was measured using standard fluorescein wavelength (λ excitation ∼ 480 nm; λ emission ∼ 530 nm). The DNA concentration in the samples was finally calculated by referencing the fluorescent signals against a standard plot. The experiment was performed in triplicate.

2.5.2. Gel electrophoresis retardation assay

In order to study the electrophoretic mobility of the DNA released from HA–PE scaffolds, the release samples collected at the established times were loaded into an agarose gel (0.7%, w/v) containing ethidium bromide (0.25 μg/ml) in TAE buffer (pH 7.2) and the electrophoresis was performed at 80 V for 45 min. The pattern of banding was visualized by a UV transilluminator and photographed by a digital camera.

2.6. Reporter gene expression assay

In a typical experiment B16-F10 cells were incubated, 24 h before transfection, in complete DMEM in 96-well plates at a density of 5000 cells/well. After 24 h, the culture medium was replaced with the pCMVLuc DNA released in PBS at pH 7.4 (in the presence or in the absence of HAase) from HA–PE scaffolds containing PE/pCMVLuc polyplexes or naked pCMVLuc plasmid and collected during the 28 days incubation period. After 4 h, the solutions in each well were replaced with fresh medium (100 μl/well) and the plates were incubated for 24 h. Then the medium was removed, the cells were washed with 200 μl of PBS and the luciferase activity was evaluated as described elsewhere (Ogris et al., 2001). Briefly, cells were washed with PBS, lysed with Promega cell lysis solution and analysed using a standard luminescence protocol. Transfection efficiency was expressed as relative light units (RLUs) per seeded cells, being 10^7 light units equivalent to 2 ng of recombinant luciferase. As a comparison, PE/DNA polyplexes (0.2 μg pCMVLuc/well; polycation/plasmid weight ratio = 4) transfection efficiency was also measured, whereas to ensure the right course of the experiment, PEI22lin/DNA polyplexes (0.2 μg pCMVLuc/well; polycation/plasmid weight ratio = 0.8) were used. The experiment was performed in triplicate.

2.7. Statistical analysis

All results are reported as mean ± standard deviation and, when applicable, statistical analysis for significance was performed by means of the Student's *t*-test (Microsoft Excel statistical function for *t*-tests) assuming unequal variance and two-tailed distribution; values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Preparation of hyaluronic acid (HA) and α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE) based scaffolds in the presence of PE/DNA polyplex

In a previous work the preparation and characterization of composite films based on hyaluronic acid (HA) and α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE) were reported (Pitarresi et al., 2008). Taking into account the promising properties in terms of biocompatibility and biodegradability showed in vitro by the HA–PE films, in the present work the efficacy of HA–PE based biomaterials as polymeric depot systems for DNA release was investigated. Considering that cell adhesion increases by increasing the PE molar amount in the scaffolds (Pitarresi et al., 2008), in the present work scaffolds containing a high molar ratio between PE amino groups and HA repeating units were produced. In particular, we prepared scaffolds as sponges and films using a molar crosslinking ratio between PE amino groups and HA repeating units (or *X* value) equal to 0.5, 1.0 or 1.5.

Preliminary studies allowed to find the optimal experimental conditions to obtain the scaffolds. In particular, both film and sponges were prepared by chemical crosslinking of HA in the presence of PE in aqueous solution of NaCl 0.9% (w/v) at pH 7.4, using N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as activators. For the films preparation, glycerol (10%, w/v) was added both to gel forming solutions and to the water used to wash the hydrogels, avoiding the shrinkage of the materials that the high PE concentration could cause during the drying phase.

In order to load DNA into the scaffolds, the crosslinking reaction between HA and PE was performed in aqueous solution containing preformed PE/DNA polyplexes with a polycation/DNA weight ratio equal to 4. This weight ratio was chosen since preliminary studies performed on PE/DNA polyplexes with a different polycation/DNA weight ratio (ranging from 0.5/1 to 25/1), demonstrated that it is the optimal weight ratio to obtain PE/DNA polyplexes with high DNA condensation ability, transfection efficiency and cell-compatibility (Cavallaro et al., 2007). In this way, scaffolds based on HA and PE containing DNA (calf thymus or pCMVLuc DNA) condensed with PE were produced by chemical crosslinking EDC/NHS activated in NaCl 0.9% (w/v) aqueous solution (Scheme 1).

HA–PE scaffold loaded with naked pCMVLuc plasmid were also prepared, in order to have a comparison for cell transfection studies. By varying the concentration of starting HA, the nominal crosslinking ratio (*X* values) and the molar amount of activators (*Y* values), as well as the hydrogel purification and drying methods, the scaffolds were obtained as films or sponges.

All prepared scaffolds were characterized to evaluate the DNA loading efficiency, microscopic morphology, cell-compatibility, DNA release ability and in vitro transfection efficiency of the released DNA.

3.2. Morphological analysis of HA–PE based scaffolds

By means of SEM analysis, morphological characterization of all HA–PE based films and sponges containing PE/DNA polyplexes was performed. The films show a quite smooth and homogeneous surface, whereas the sponges show a highly porous structure with interconnected pores with a mean diameters which ranged from about 110 to 350 μm, for sample with $X = 1.5$ and $X = 0.5$, respectively. Both the two kinds of materials show properties encouraging for a potential use in tissue engineering applications: films are probably more suitable as bioactive membranes for local gene therapy in wound dressing/healing (Kim et al., 2005), but sponges could be useful as three-dimensional structures for tissue regeneration.

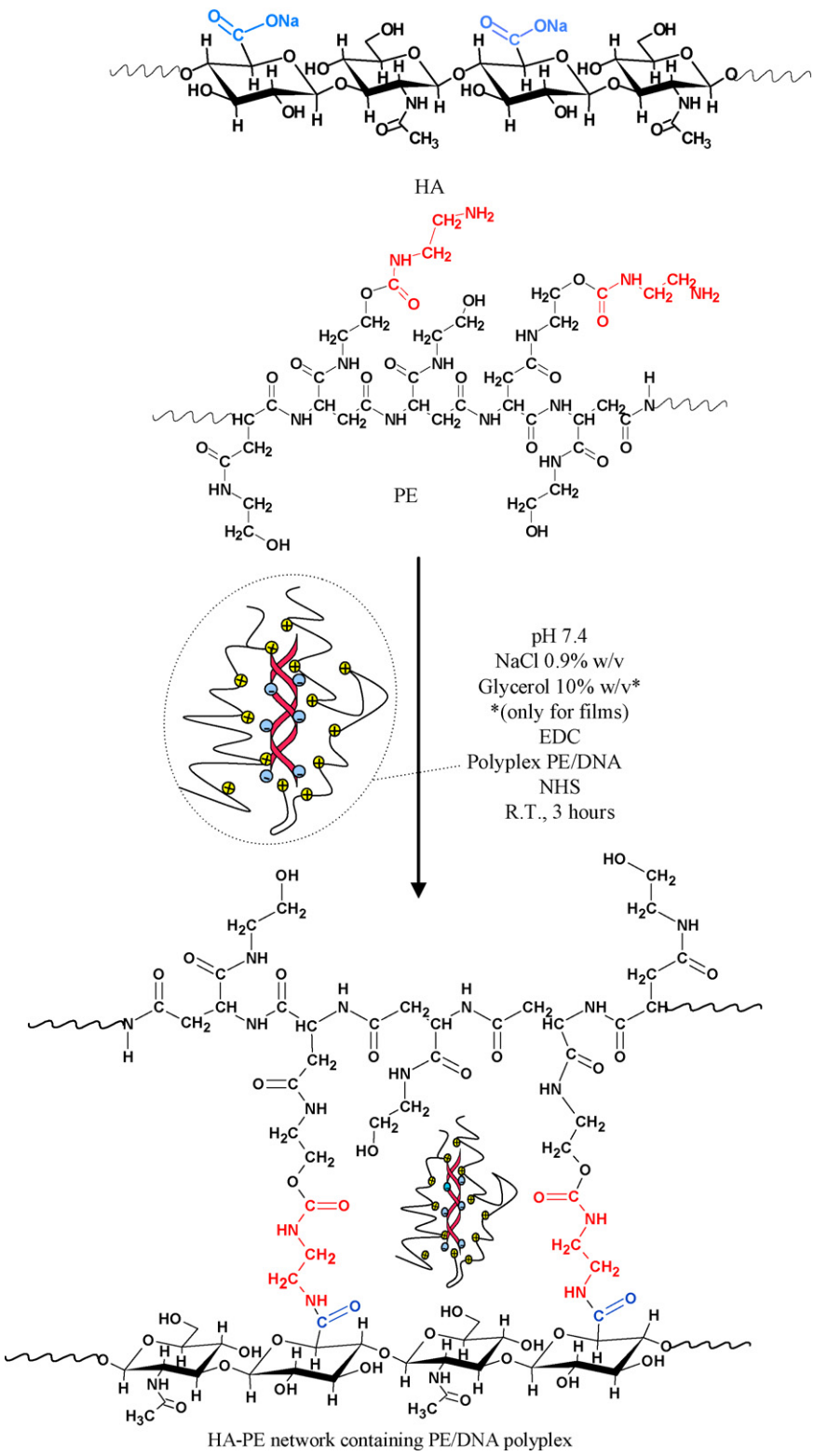
3.3. Cell-compatibility studies

The biocompatibility of HA–PE scaffolds containing PE/DNA polyplexes was studied in vitro on human dermal fibroblasts. The viability of the cells incubated in direct contact with HA–PE based films and sponges unloaded or loaded with PE/DNA polyplexes was determined by means of MTS assay after 48 h of incubation and the results are reported in Fig. 1.

Moreover, in order to investigate the cell-compatibility of potential degradation products of the HA–PE based films and sponges, an indirect assay was also performed. The viability of human dermal fibroblasts after 48 h of incubation in culture medium where HA–PE films and sponges have been previously swelled for 12 or 24 days ("conditioned medium"), was investigated by MTS assay. Data thus obtained are reported in Fig. 2. It is evident that the investigated samples do not cause significant variation in cell viability after their direct contact with the cells, and their degradation products do not negatively interfere with cell viability, so that these materials do not contain significant amounts of biologically harmful extractable.

3.4. DNA release studies

In order to compare the DNA release profile from the different scaffolds, DNA delivery studies were firstly performed using HA–PE based films and sponges ($X = 0.5$, 1.0 or 1.5) loaded with PE/calf thymus DNA polyplexes. In particular, circular samples of films and sponges were incubated at 37 °C in PBS pH 7.4 in the absence or in the presence of HAase (10 U/ml), in order to evaluate the effect of the enzyme on the DNA delivery trend. DNA cumulative per-



Scheme 1. Crosslinking reaction between HA and PE in the presence of PE/DNA polyplexes.

cent release data obtained by means of the spectrofluorimetric Pico Green assay, are reported in Fig. 3.

As it can be observed, during the 28 days incubation all the scaffolds release DNA in a different way depending on both the presence of enzyme and the crosslinking ratio *X*. Since second day of incubation, the amount of DNA released from each sample in the presence of HAase is always significantly higher than that obtained in the absence of the enzyme ($p < 0.05$), this result indicates that enzymatic degradation of the scaffolds promotes the DNA release. As previously demonstrated, HA–PE scaffolds are degraded by HAase (Pitarresi et al., 2008) which is able to hydrolyse the network acting on the polysaccharide moiety. This event

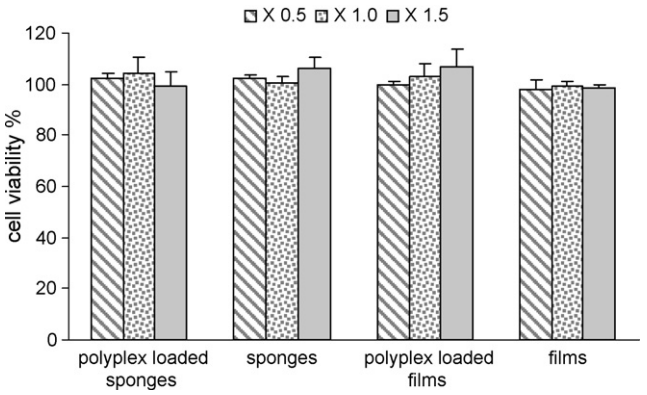


Fig. 1. Human dermal fibroblasts viability % (percentage of control) after 48 h of incubation (100,000 cells/ml in complete MEM) in the presence of HA–PE based scaffolds unloaded or loaded with PE/DNA polyplexes.

would reduce the ability of the matrices to retain the DNA complexed with the PE allowing its release in the aqueous medium. The amount of DNA released depends also on the relative molar amount of PE in the material: DNA release a significantly increases ($p < 0.05$) by increasing the ratio between the amino groups of PE and the carboxyl groups of HA (indicated as X value). This result can be explained considering a different ability of HA–PE based scaffolds to keep in the polyplex PE/DNA by electrostatic interactions, which is dependent on the X value. In fact, PE/DNA polyplexes with a polycation/plasmid weight ratio equal to 4, having a positive charge as revealed by zeta potential studies (Cavallaro et al., 2007), could interact electrostatically with the free carboxyl groups present into the HA–PE networks, which at pH 7.4 are on the dissociated form. The latter interaction decreases by increasing X value since the number of carboxylate groups decreases, consequently DNA release increases with the following order: $X = 1.5 > X = 1.0 > X = 0.5$, ranging from a minimum of $10.0 \pm 0.6\%$ of DNA loaded, obtained for $X = 0.5$ sponge incubated in the absence of HAase, to a maximum of $95.1 \pm 0.1\%$ for the sponge $X = 1.5$ incubated in the presence of HAase.

Since the HA–PE based film and sponge with $X = 1.5$ were able to release more DNA than scaffolds with a lower X value, they were selected for the further studies. The DNA release from HA–PE films and sponges with $X = 1.5$ containing PE/pCMVLuc polyplexes was studied by means of the spectrofluorimetric Pico Green assay, performed as reported above, and obtained data are reported in Fig. 4.

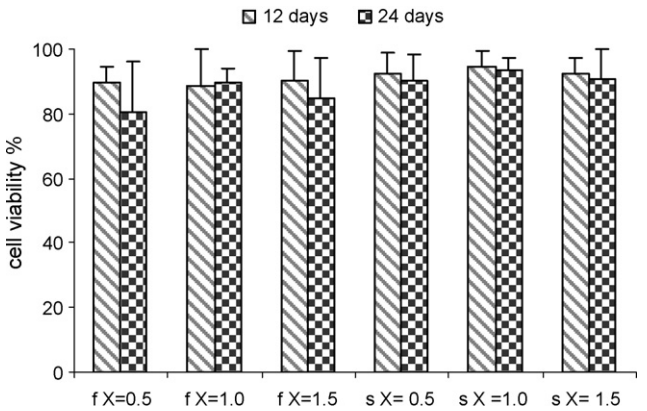


Fig. 2. Human dermal fibroblasts viability % (percentage of control) after 48 h of incubation (100,000 cells/ml) in MEM conditioned with HA–PE films (f) or sponges (s) for 12 or 24 days.

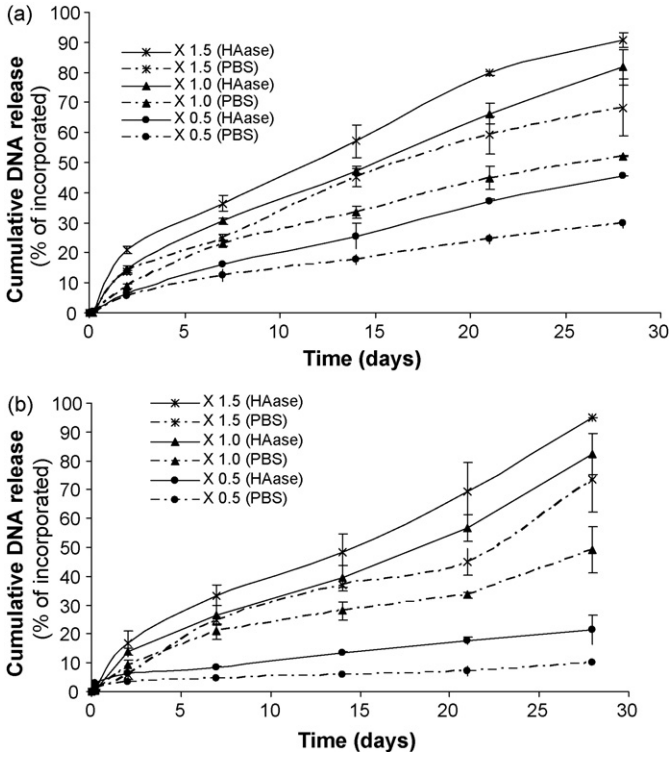


Fig. 3. In vitro cumulative DNA (calf thymus) release from polyplex loaded HA–PE films (a) and sponges (b) incubated at 37 °C in PBS pH 7.4 ± HAase (10 U/ml); data are reported as percent weight ratio between released and loaded DNA as a function of time.

3.5. Electrophoresis retardation assay

Gel electrophoresis retardation assay was performed on the samples of DNA released from HA–PE based scaffolds and collected during the 28 days of incubation. In the pictures reported in Fig. 5 it can be observed that for all the samples the fluorescence is mostly in the wells, thus suggesting that DNA released is not naked but it is probably strongly complexed with a polymer that blocks its migration during the electrophoresis. Since the banding pattern obtained for the investigated samples is comparable to that obtained with free PE/DNA polyplexes, we hypothesized that DNA is released from the scaffolds as a complex with the PE.

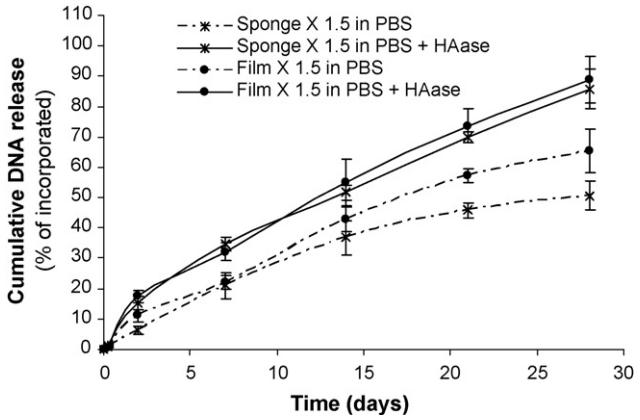


Fig. 4. In vitro cumulative DNA (pCMVLuc) release from polyplex loaded HA–PE films or sponges ($X = 1.5$) incubated at 37 °C in PBS pH 7.4 ± HAase (10 U/ml); data are reported as percent weight ratio between released and loaded DNA as a function of time.

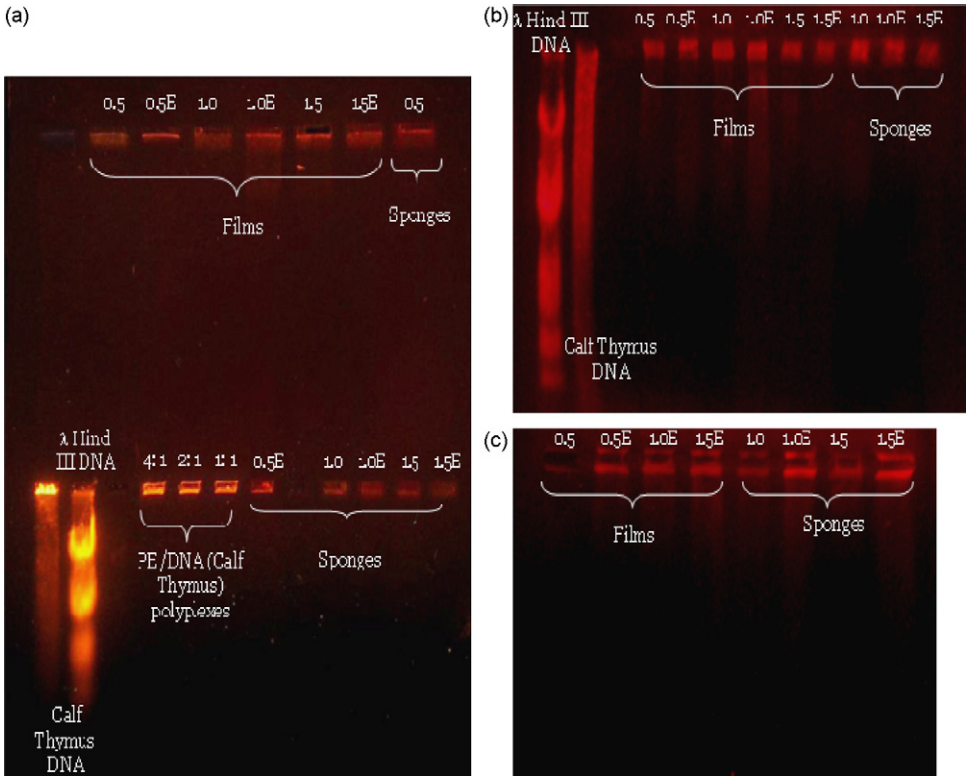


Fig. 5. Electrophoretic analysis in gel of agarose (0.7%, w/v) performed on the release medium ± HAase (the presence of the enzyme is indicated with "E") collected after 14 days (a), 21 days (b) and 28 days (c) incubation with HA–PE films or sponges containing PE/DNA polyplexes. As a control, the electrophoretic mobility of PE/DNA polyplexes (in weight ratio equal to 4:1, 2:1 and 1:1) and of naked DNA is also reported.

3.6. Cell transfection studies

In order to evaluate the structural integrity and biological activity of DNA released from HA–PE based scaffolds, cell transfection studies were performed in vitro on B16-F10 cells chosen as a model line, using luciferase enzymatic activity as reporter gene expression. In particular, transfection efficiency of the plasmid released from HA–PE films and sponges ($X = 1.5$) loaded with PE/pCMVLuc polyplexes or naked pCMVLuc, and collected throughout 28 days of incubation at 37 °C in PBS pH 7.4 in the absence or in the presence of HAase (10 U/ml) was evaluated. Data thus obtained (Fig. 6) show that the scaffolds loaded with polyplexes release biologically active DNA in the conditions of incubation. Since naked DNA is not able to pass easily through plasmatic or nuclear membranes, it is reasonable to suppose that DNA released from the scaffolds could be efficaciously internalised and transfected by the treated cells only if condensed with PE. This hypothesis is confirmed by the negligible transfection efficiency values obtained for scaffolds loaded with naked DNA (also reported in Fig. 6), thus indicating the significance of polyplexes formation before the incorporation into the scaffolds. Moreover, any significantly difference in the transfection efficiency was observed between DNA released from films and sponges both in the presence and in the absence of HAase, probably suggesting that DNA structural integrity or its biological activity are not affected by the incorporation method, by the presence of HAase or by degradation products potentially released from the scaffold in the release medium. Even if the transfection efficiency of DNA released from HA–PE scaffolds is lower than that obtained from PEI/DNA polyplex (used as a positive control), for a use in the field tissue engineering, the possibility to obtain a prolonged and local delivery of genetic material compensates for the lower transfection efficiency. On the other hand, unlike PEI, which cytotoxicity is known (Wightman et al., 2001), PE is biocompatible as elsewhere reported (Cavallaro et al., 2007).

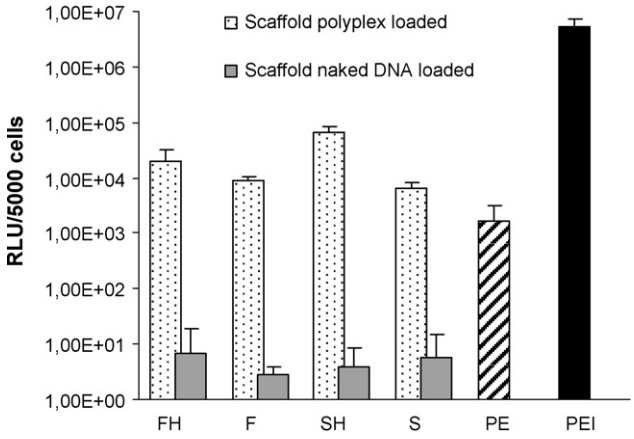


Fig. 6. Transfection efficiency (luciferase assay) in B16-F10 cell line (5000 cells/well) of DNA released from HA–PE films (F) and sponges (S) containing PE/pCMVLuc polyplex or naked pCMVLuc plasmid incubated in PBS pH 7.4 in the absence or in the presence of HAase (10 U/ml, indicated as "H") for 28 days. Results obtained using PE/pCMVLuc polyplex (indicated as PE) (0.2 μg pCMVLuc/well; polycation/plasmid weight ratio = 4) and PEI/DNA polyplex (indicated as PEI) (0.2 μg pCMVLuc/well; polycation/plasmid weight ratio = 0.8) are also reported.

4. Conclusions

This study reports a successful method to produce composite biocompatible scaffolds, shaped as films or sponges, for a potential use for local gene delivery. By means of EDC/

NHS chemistry, hyaluronic acid (HA) was crosslinked with a polyaspartamide derivative, the α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PE), in the presence of a model DNA previously complexed with PE, as a biocompatible polymeric vector. In this way, novel HA–PE films and sponges with high PE/DNA polyplex loading were produced and they showed promising properties for a potential application as depot systems for a controlled DNA release. DNA delivery studies revealed that HA–PE scaffolds release progressively DNA as a complex with the PE, with a trend which is dependent on the presence of hyaluronidase and on the molar ratio between PE and HA. In vitro cell transfection studies demonstrated that the released DNA preserves its biological activity, being able to transfect the treated cells as well as the free PE/DNA polyplexes. These results demonstrate that the method used in this work to load DNA into the scaffolds is highly efficient (the DNA loading efficiency was more than 71%) and it does not damage DNA which maintains its biological activity during all the steps of synthesis, purification and release. Further studies will be performed in order to evaluate the efficacy of HA–PE based scaffolds as gene activated matrices for tissue engineering applications.

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