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Functional Single-Chain Polymer Nanoparticles:

Targeting and Imaging Pancreatic Tumors *in vivo*

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ABSTRACT. The development of tools for the early diagnosis of pancreatic adenocarcinoma is an urgent need in order to increase treatment success rate and reduce patient mortality. Here, we present a modular nanosystem platform integrating soft nanoparticles with a targeting peptide and an active imaging agent for diagnostics. Biocompatible single-chain polymer nanoparticles (SCPNS) based on poly(methacrylic acid) were prepared and functionalized with the somatostatin analog PTR86 as the targeting moiety, since somatostatin receptors are over-expressed in pancreatic cancer. The gamma emitter ^{67}Ga was incorporated by chelation and allowed *in vivo* investigation of the pharmacokinetic properties of the nanoparticles using Single Photon Emission Computerized Tomography (SPECT). The resulting engineered nanosystem was tested in a xenograph mouse model of human pancreatic adenocarcinoma. Imaging results demonstrate that accumulation of targeted SCPNS in the tumor is higher than that observed for non-targeted nanoparticles due to improved retention in this tissue.

INTRODUCTION

Cancer accounted for 8.2 million deaths worldwide in 2012¹ and for some types of cancer, e.g. pancreatic adenocarcinoma (78.000 deaths in Europe in 2012²), incidence equals mortality.³ Most pancreatic adenocarcinomas are still detected at a late stage, and 85% are unresectable at the time of detection.⁴ This is due, in part, to the lack of appropriate tools for early detection, accurate staging and post-therapy monitoring. The maturity of *in vivo*-minimally invasive imaging methods together with the development of novel nanomaterials-based imaging probes has posed a great expectation in the field of *in vivo* cancer detection and monitoring.⁵ As a consequence, the design and development of novel nanosystems integrating advanced nano-sized

core particles which can be functionalized with targeting (bio)molecules and imaging agents is currently an issue of intense research.^{5,6}

Nanoparticles (NPs) can profit of passive tumor-targeting due to the well-known enhanced permeation and retention (EPR) effect.⁷ The size of the NPs is a key parameter for the NPs to access the tumor tissue, and this is particularly critical in the case of poorly permeable tumors, such as intractable pancreatic adenocarcinoma, where NPs with diameters above 100 nm display lower penetration and accumulation than sub-100 nm NPs.^{8,9,10} Additionally, surface decoration of the NPs using biomolecules capable to target specific biomarkers over-expressed in tumor cells, such as peptides or antibodies, can be used to improve selective or preferential accumulation in the tumors via active targeting,^{11,12} at the cost of adding complexity to the NPs while increasing particle size. The use of peptides is usually preferable due to a better chemical control both on formulation and further functionalization, lower cost and easier manipulation.¹³ Of note, the conjugation of peptides to nanoparticles usually results in an improved biological half-life with respect to the free peptide and an enhanced affinity for their target thanks to multivalent interactions.^{14,15}

In the pursuit of molecularly targeted imaging nanostructured agents, different examples of NPs bearing targeting peptides and different imaging probes have been reported in the literature.^{16,17} Weissleder and collaborators¹⁸ conjugated targeted peptides to magnetofluorescent NPs based on cross-linked iron oxides for pancreatic ductal adenocarcinoma detection. More recently, pancreatic tumor receptor-targeted magnetic iron oxide nanoparticles were developed as a magnetic resonance imaging (MRI)-based theranostic nanosystem.¹⁹ While MRI is one of the most commonly used non-invasive imaging techniques in the clinical setting and has proven powerful in the visualization of the pancreas,²⁰ it has a severe limitation in terms of sensitivity;

additionally, quantification of the images results extremely challenging. As a consequence, incorporation of positron or gamma emitters into the NPs to enable subsequent tracking *in vivo* using ultra-sensitive nuclear imaging techniques such as Positron Emission Tomography (PET) or Single Photon Emission Computerized Tomography (SPECT) in combination with anatomical techniques, such as computerized tomography (CT) has recently gained significance.^{21,22,23,24}

In this work, we present the synthesis of water-dispersible, single-chain polymer nanoparticles (SCPNs), their functionalization with a targeting peptide, the subsequent radiolabeling with a gamma emitter, and their evaluation in a pre-clinical model of pancreatic adenocarcinoma. SCPNs are based on the controlled collapse of single polymer chains into folded nanoparticles by intra-chain cross-linking, which leads to defined nanostructures that can mimic the folding process of biomolecules.^{25,26} Fine tuning of the size can be achieved by controlling the molecular weight of the precursor polymer chain and the quantity of intramolecular bonds generated in the collapse.²⁷ Additionally, accurate selection of the starting materials can ensure biocompatibility/biodegradability and facilitates surface decoration. In our case, a newly developed methodology enabled the preparation of poly(methacrylic acid) (PMAAc)-based, <50 nm-sized and water dispersible SCPNs in mild conditions: room temperature, water, and absence of metal catalysts. The synthetic somatostatin analogue PTR86, a peptide with high affinity towards somatostatin receptors 2,3,4 and 5 (SSTR2-5, highly expressed in pancreatic tumors²⁸), was anchored to the carboxylic acid groups at SCPNs surface to achieve non-toxic, targeted NPs. Diethylenetriaminepentaacetic acid (DTPA)-containing PMAAc SCPNs could be loaded with Gallium-67 (⁶⁷Ga) for SPECT imaging. After radiolabeling, *in vivo* imaging experiments using the targeted SCPNs were performed in mice bearing sub-cutaneous human pancreatic ductal adenocarcinoma (PANC-1) tumors using SPECT in combination with CT, and results were

compared to non-targeted SCPNs and the free PTR86 peptide as controls. Our results show improved accumulation of the targeted PMAAc SCPNs in the tumor and suggest their potential application as diagnostic agents.

MATERIALS AND METHODS

Synthesis of SCPNs

Materials. All chemicals were used as received, except otherwise noted. Picoline borane complex (95%) (CAS: 3999-38-0), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM·HCl) (96%) (CAS: 3945-69-5) and dimethyl sulfoxide (DMSO) (98%) (CAS: 67-68-5) were purchased from Aldrich. Acetone, technical grade, (CAS: 67-64-1) was purchased from Scharlau. Water (H₂O) used in the syntheses, unless otherwise stated, was deionized water from a MilliQ A10 Gradient equipment (Millipore). PTR86 (PTR 3207-86) derivative was synthesized as previously described.²⁸ The *N*-terminus of PTR86 was equipped with γ -aminobutyric acid (GABA) to generate free amino group available for the conjugation to fluorescein isothiocyanate (FITC) or to 1,4,7-triazacyclononane-1,4,7-trisacetic acid (NOTA) chelating agent, whereas NP was conjugated through amine pendant group from lysine.

Synthesis of PMAAc SCPNs (3). Cross-linker **2** and copolymer **1** (Scheme 1) were synthesized as described in the SI. In order to prepare the PMAAc SCPNs (**3**), copolymer **1** (840 mg) was suspended in distilled water (20 mL) and dispersed by treating with a saturated solution of aqueous NaOH (1 mL). The pH was then adjusted to ~6 with aqueous HCl 1M, the volume was raised to 55mL with water (final concentration of the reactive monomer around 0.02 M). Cross-linker **2** (0.18 M solution, 1.75 mL) was then added using a syringe pump at a constant rate of 0.82 mL/h at room temperature under stirring. After that, picoline borane (solid, 116 mg) was

added. The reaction mixture was maintained at room temperature overnight under stirring and subsequently added dropwise over cold acetone (500 mL) under vigorous stirring. After 5 hours at 4°C, the precipitate was separated from the liquid and dried to obtain a white solid (790 mg), which was used without further purification. Dialysis against water (Snake Skin Pierce membrane, MWCO: 3.5 kD, against 4.5 L of Milli-Q water, refreshing 3 times per day over the course of 72 h) of a small aliquot of the crude (25mg) afforded pure PMAAc SCPNs (**3**) (12 mg).

Synthesis of PTR86-functionalized PMAAc SCPNs (PTR86-PMAAc SCPNs, 5). The nanoparticles crude (60 mg) prepared as described above was dispersed in water (1 mL, 29 mg/mL of PMAAc SCPNs (**3**), calculated from a dialyzed aliquot) and the pH was adjusted to pH 8.0-8.5 by addition of aqueous NaOH 1M. The final volume was raised to 2 mL with water and then the mixture was treated with a water solution of DMTMM·HCl (100 mg/mL, 325 µL). After stirring for 20 minutes at room temperature, a solution of fluorescently labeled PTR86 (FITC-PTR86, **4**) in DMSO (17.2 mg/mL, 116 µL) was added. The mixture was stirred at room temperature overnight. The resulting nanoparticles were purified by dialysis (Snake SkinTM membrane, MWCO: 3.5 kDa, against 4.5 L of Milli-Q water). The system was permanently protected from light. The content of the beaker was stirred slowly, refreshing with Milli-Q water 3 times per day over the course of 72 h. After dialysis, PTR86-PMAAc SCPNs (8 mL 3.4 mg/mL) were obtained and stored at 4°C. The concentration was determined by freeze-drying 2 mL of sample which yielded a white solid (6.7 mg).

Nuclear Magnetic Resonance (NMR). ¹H NMR spectra were recorded at 25°C on a Bruker AVANCE III spectrometer working at 500 MHz for hydrogen nuclei.

Gel permeation chromatography (GPC). GPC analyses were performed on a PL-GPC 50GPC Integrated GPC/SEC System (Agilent) equipped with 2 Agilent columns sequence (a PL-

aquagel-OH Guard 8 μm 50x7.5 mm pre-column and a PL-aquagel-OH MIXED-M 8 μm 300x7.5 mm column) and a refractive index detector. 500 μL of a 5 mg/mL dispersion of the sample (PMAAc co-polymer **1** or the corresponding PMAAc SCPNs **3**) in 0.05 M TRIS buffer (pH 7.3) with 0.2 M NaNO_3 were injected into the column (eluent: same as the dispersion buffer; loop: 200 μL ; flow rate: 1 mL/min; temperature: 40 $^\circ\text{C}$).

Dynamic Light Scattering (DLS). The hydrodynamic radius of the SCPNs was measured on a Zetasizer Nano ZS, ZEN3600 Model (Malvern Instruments Ltd) by DLS (NIBS[®]) technique. All measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 173 $^\circ$. A filtered (0.22 μm) water dispersion of the nanoparticles at a concentration of 0.3 mg/mL was used and the experiments were performed at 25 $^\circ\text{C}$ in triplicate.

Transmission electron microscopy (TEM). TEM analyses were performed in a TECNAI G2 20 TWIN microscope (FEI, Eindhoven, The Netherlands), operating at an accelerating voltage of 200 KeV in a bright-field image mode. One drop of the sample dispersion in water (~ 3 μL , 0.035 mg/mL) was deposited on a carbon film supported on a copper grid (300 mesh), hydrophilized by a glow discharge process just prior to use. After staining for 20 seconds with uranyl acetate aqueous solution (1% w/v), the sample was rotated at high speed in order to dry at room temperature quickly by spinning process. Size and size distribution was measured by imageJ analysis of 300 counts.

Fluorescence Spectroscopy. Fluorescence measurements were performed in a Cary Eclipse Fluorescence Spectrophotometer (Varian) to estimate the amount of FITC-PTR86 peptide immobilized on the SCPNs (excitation wavelength: 490nm; emission range wavelength: from 495nm to 600nm). Fluorescence intensity of the peak at 521nm was used for measurements. A calibration curve was obtained using peptide PTR86 as standard (see Figure S14).

Synthesis of PTR86 peptide derivatives

PTR86 and its fluorescently-labelled derivative FITC-PTR86 (**4**) were synthesized consisting of a backbone cyclic core peptide, $\text{NH}_2\text{-GABA-(D)Phe-cyclo(Cys-Phe-Trp-(D)Trp-Lys-Thr-Phe-GlyS2)-amide}$ following a previously described procedure.²⁸ Building block (GlyS2) bearing thiol functional group was synthesized according to Gazal *et al.*²⁹ Coupling of the peptide to NOTA chelating agent was performed according to previously described methods^{29,30,31} (see SI). 25 mg of PTR86-NOTA (**6**) were obtained (15% overall yield).

Radiolabeling

Radioactive material. Gallium-67 (^{67}Ga , $T_{1/2} = 3.26$ days) was selected as the radioisotope because of its long half-life, which enabled longitudinal follow-up of the biodistribution pattern up to several days. Gallium-67 was purchased as citrate solution from Molypharma S.L. (Biscay, Spain), and was converted into $^{67}\text{GaCl}_3$ using a solid-phase extraction method.³² Briefly, the citrate solution was eluted through two C18 Sep-Pak silica plus light cartridges (Waters chromatography S.A., Massachusetts, USA). The cartridges were washed with ultrapure water (obtained from a Thermo Scientific Barnstead NANOpure Diamond Water System, Thermo Fisher Scientific, Waltham, MA, USA) and the radionuclide was then desorbed by eluting with 0.1 M HCl (1 mL) prepared by dilution of $\text{HCl}_{30\%}$ (Suprapur[®], Merck kGaA, Darmstadt, Germany) with ultrapure water. The eluate was collected in 10 different fractions of 100 μL each, and only those containing the maximum activity concentration were used in subsequent labeling experiments.

Radiolabeling of SCPNs. Radiolabeling of SCPNs was performed by incubation of the SCPNs with a solution of $^{67}\text{GaCl}_3$; briefly: 200 μL of a 0.2 M sodium acetate buffer solution (pH 3.8) was mixed with 50 μL of PMAAc SCPNs (**3**) or PTR86-PMAAc SCPNs (**5**) solution (1 mg/mL)

and 100 μL $^{67}\text{GaCl}_3$ (0.80 ± 0.02 MBq -370 MBq). The mixture was incubated at room temperature for 30 minutes. The crude material was then purified by centrifugal filtration using a Nahita 2507/100 centrifuge (Auxilab S.L.) and Millipore filters (10 kDa cut-off, Amicon Ultra-0.5 mL UFC510096, Molsheim, France). After centrifugation (8 minutes, 2862 g) the precipitate was washed twice with sodium acetate buffered solution to remove unreacted ^{67}Ga , and the amount of radioactivity in the pellet, the supernatant and the washings was determined in a dose calibrator (CRC® - 25R, Capintec, Inc.). Labeling efficiency (expressed in percentage) was calculated as the ratio between the amount of radioactivity in the filter and the total amount of radioactivity in all fractions. Finally, SCPNs were suspended in 200 μL of saline solution (0.9% NaCl, Braun Medical S.A., Catalonia, Spain). Radiochemical yield was calculated as the ratio between the amount of radioactivity in the resuspended fraction and the starting amount of radioactivity.

Stability assays of the radiolabeled SCPNs. To assess the radiochemical stability of the NPs *in vitro*, SCPNs were prepared and purified as described above and subsequently suspended in 500 μL of physiological NaCl 0.9% solution. The suspensions were then divided into 5 different 100 μL aliquots. The aliquots were kept at 37°C for 2 minutes, and 1, 3, 24 and 48 hours, respectively, using a digital block heater. The samples were then filtered and radioactivity in the filter and the filtrate was measured in a 2470 WIZARD² Automatic Gamma-counter (Perkin-Elmer). The radiochemical integrity of the NPs, expressed in percentage, was calculated as ratio between the amount of radioactivity in the filter and the amount of radioactivity in both fractions (filter + filtrate).

Radiolabeling of PTR86-NOTA (6). To a solution containing sodium acetate buffer 0.2 M (200 μL) and $^{67}\text{GaCl}_3$ (100 μL , 0.012 ± 0.002 MBq 296-370 MBq), a solution of PTR86-NOTA (100

μL , 1 mg/mL in DMSO, total amount = 100 μg) was added, and the vial was heated at 70 °C for 15 minutes. The reaction crude was then cooled at room temperature and ultrapure water (5 mL) was added. The resulting solution was eluted through a C-18 cartridge, where the labeled peptide was retained. The peptide was then eluted with 1 mL EtOH and collected in a conic vial. The ethanol was evaporated to a final volume of c.a. 50 μL under a stream of N_2 , and the resulting solution was finally reconstituted with physiological saline solution to a final volume of 500 μL . Radiochemical purity was assessed by HPLC using an Agilent 1200 Series HPLC system with a multiple wavelength UV detector ($\lambda = 220 \text{ nm}$) and a radiometric detector (Gabi, Raytest) connected in series. A RP-C18 column (MediterraneaTM Sea18, 4.6x150 mm; Teknokroma, Spain) was used as stationary phase and solutions A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile) were used as the mobile phase. The following gradient was applied: from $t = 0\text{-}2 \text{ min}$, 30% B; from $t = 2\text{-}10 \text{ min}$, linear gradient to reach 80% B; from $t = 10\text{-}12 \text{ min}$, 80% B; from $t = 12\text{-}13 \text{ min}$, linear gradient to reach 30% B; from $t = 13\text{-}20 \text{ min}$, 30% B. Injected volume was 20 μL and retention time for the labeled peptide was 8.6 min. Radiochemical purity was above 95% at injection time in all cases.

Stability assays of the radiolabeled PTR86-NOTA (6). Radiolabeled PTR86-NOTA was prepared and purified as described above. A 50 μL fraction was diluted to 500 μL using physiological NaCl 0.9% solution. The solutions were then divided into 5 different 100 μL aliquots. The aliquots were kept at 37°C for 2 minutes, and 1, 3, 24 and 48 hours, respectively, using a digital block heater. The samples were then analyzed by HPLC using the method described above. The stability of the labeled peptides was determined from chromatographic profiles, as the ratio between the area under the peak corresponding to the labeled peptide and the sum of the areas for all peaks in the chromatogram.

In vivo imaging studies

Pancreatic tumor cell line. The human pancreatic tumor cell line PANC-1 was obtained from the European Collection of Cell Cultures (ECACC Cell Lines, Sigma Aldrich). PANC-1 cells were maintained as monolayer culture in RPMI-1640 (Lonza, Verviers, Belgium) with 10% Fetal Calf Serum (FCS, Sigma-Aldrich, St. Louis, USA), 1% streptomycin/penicillin (Invitrogen, USA) and 2 mM glutamine (Lonza, Verviers, Belgium). Cultures were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air. Regular assessment of the presence of Mycoplasma was performed with the use of a commercial kit (Lonza, Rockland, ME, USA).

Animal model. All animal procedures were performed in accordance with the Spanish policy for animal protection (RD53/2013), which meets the requirements of the European Union directive 2010/63/UE. All experimental procedures were approved by the Ethical Committee of CIC biomaGUNE and authorized by the regional government under the protocol code AE-biomaGUNE-0813. Mice were housed in polycarbonate ventilated cages (Tecniplast) under a controlled environment (12 hours/12 hours light/dark cycle with dawn and dusk transitional periods, room temperature 22 ± 2°C and relative humidity 55 ± 10%) and maintained on an *ad libitum* access to commercially available pelleted diet (Teklad 2919, Harlan Laboratories, Inc.) and sterilized water. For the generation of the animal model, PANC-1 cells were centrifuged and the pellet was subsequently re-suspended in Dulbecco's Phosphate Buffered Saline (PBS) (Lonza, Verviers, Belgium) at a concentration of 2x10⁷ cells/mL. The cell suspension was mixed with Matrigel (Becton Dickinson, Oak Park, Bedford, MA) at a 1:1 (v/v) ratio and kept at 4°C. Male athymic-nude CD-1 Foxn1 nu/nu mice (Charles River Laboratories, Calco, Italy) were injected subcutaneously with 2 x 10⁶ PANC-1 cells (200 µL of above mentioned mixture) in the upper left flank. Frequent monitoring of tumor volume and body weight was performed for the

whole duration of the experiment. The tumor volume was calculated using the formula $(L \times W^2)/2$ where L (in mm) is the longest diameter of the tumor and W (in mm) is the longest perpendicular diameter with respect to L. Both dimensions were determined with a caliper (LAWTON GmbH & Co. KG). Imaging sessions were performed when tumor volumes reached a volume above 150 mm³ (approximately after 8-10 weeks post-inoculation of the cells).

SPECT. Biodistribution studies were performed in mice bearing subcutaneous PANC-1 tumors (*vide supra* for animal model generation). Three hours before the examination, mice (n=5 per labeled species) received an intravenous administration of the radiolabeled nanoparticles (300 µL, 44.4 ± 13.6 MBq of PMAAc SCPNs or 24.7 ± 6.6 MBq of PTR86-PMAAc SCPNs, approximately 30 µg of NP) or peptides (300 µL, 17.3 ± 0.3 MBq, approximately 10 µg of peptide). During the administration of the doses and image acquisition, sedation was first induced using 4.5 - 5% isoflurane and maintained with 1.5 - 2% isoflurane blended with O₂. Images were acquired using an eXplore speCZT CT preclinical imaging system (GE Healthcare, USA). Body temperature was maintained using a homeothermic blanket control unit (Bruker BioSpin GmbH, Karlsruhe, Germany) to prevent hypothermia. Whole-body SPECT-CT scans were acquired at 3, 24 and 48 hours post-injection for 30 minutes, 60 minutes, or 120 minutes, respectively. The image acquisition period was dependent on the counts detected by the collimator of the scanner; longer imaging times were needed as the gamma emissions decreased. After each SPECT scan, CT acquisitions were performed to provide anatomical information of each animal. The SPECT images were reconstructed using a ordered-subset expectation maximization (OSEM) iterative algorithm (3 iterations/5 subsets, 128 x 128 x 32 array with a voxel size of 0.4 x 0.4 x 2.46 mm³), whereas for the CT image reconstruction, a cone beam filtered back-projection a Feldkamp algorithm (437 x 437 x 523 array with a voxel size of

0.2 x 0.2 x 0.2 mm³) was used. Image quantification was performed using PMOD analysis software (version 3.4, PMOD Technologies Ltd.). SPECT images were co-registered with CT images; the volumes of interest (VOIs) were drawn around the tissues under investigation (tumor and muscle), and tumor-to-muscle (T/M) ratios were calculated.

Statistical data analysis. For *in vivo* determination of tumor-to-muscle ratios, statistical analysis was performed using GraphPad Prism (version 5.04, GraphPad Software, Inc.). Differences within each group at different time points were analyzed using a paired, two-tailed Student's t-test; differences between groups at each time point were analyzed using an unpaired, two-tailed Student's t-test. Statistical significance was considered at $P < 0.05$.

Toxicity studies

In vitro toxicity. PMAAc SCPNs were tested for cellular toxicity on the pancreatic adenocarcinoma derived cell line PANC-1, used for *in vivo* SPECT experiments, and additional pancreatic adenocarcinoma cell lines: BxPC3, Su86.86, Colo-357, ASPC-1, and T3M4. *In vitro* toxicity was assayed by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Cell viability was evaluated in triplicate after 72 hours of incubation with the SCPNs at different concentrations.

In vivo toxicity. Acute toxicity was tested on male CD-1 (ICR)mice (Charles River, Calco, Italy) that were intravenously injected with different doses (100 mg/kg; 25 mg/Kg; 12,5 mg/Kg) of a 10 mg/mL suspension of PMAAc SCPNs. Three or five (for 12.5 mg/kg treatment) animals per group were used. As control, a group of animals was treated with 0.9% NaCl saline solution (two mice). The mice were monitored at 5 min, 15 min, 30 min, and 1 hour post-treatment. Side effects were monitored by behavioral, neurological, and other observations according to a standardized observation grid derived from Irwin.³³ Twenty-five items were evaluated and

scored using a points system ranging from 0 to 8. Animals were first placed in a transparent box and observed for behavioral changes and neurological manifestations, and then examined to evaluate reflexes and other clinical signs. Twenty-four hours later, the mice were weighed, euthanized, and submitted to complete necropsy. Liver, spleen, and kidneys were sampled and weighed. The relative organ weights (expressed as percentage) were calculated as wet organ/total body weight. Histological analysis was carried out by fixing the organs in 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm thick sections. Thereafter, the sections were stained with hematoxylin–eosin and examined for histological lesions. Blood samples were collected at the sacrifice by cardiac puncture for hematology and serum chemistry. Samples were counted with a laser based counter (Sysmex-XT 2000iV) using a species-specific software. A blood smear was prepared from each sample, then samples were centrifuged and plasma (350 to 650 μLs) was transferred in another tube and frozen at -20° . Leukocyte differential, morphology of blood cells, and platelet estimate were determined on May Grünwald-Giemsa stained smears. Clinical chemistry was performed on thawed plasma samples with an automated spectrophotometer (ILAB-300, Instrumentation Laboratory). All procedures were conducted in accordance with a protocol approved by the local authorities according to the national legislation (D.L.vo 116/92, Italy).

RESULTS AND DISCUSSION

Synthesis and functionalization of PMAAc SCPNs (3). SCPNs can be obtained by different synthetic routes, including covalent homo-functional or hetero-bifunctional single polymer chain collapse and cross-linking collapse.^{25,34} The intramolecular contraction of polystyrene by

Friedel-Crafts reaction with 1,2-dichloroethane as cross-linker was achieved in ultra-diluted solution more than 30 years ago by Martin and Eichinger.³⁵ In order to set up multi-gram scale preparations, Hawker's group³⁶ overcame the limitation of ultra-dilution conditions by means of "continuous addition" methodology: slow addition of a benzocyclobutene-functionalized linear polystyrene to a heated organic solvent promoted the controlled intramolecular collapse avoiding inter-chain reticulation. Recently, Qiao's group reported that SCPNs can be formed at high polymer concentration (ca. 100 mg mL⁻¹) by covalent chemistry (organocatalyzed ring-opening polymerization, in this case) when using polymers with side-chain functionalities like oligo(ethylene glycol) brushes which presumably prevent intermolecular cross-linking by shielding the single polymer chains.³⁷ Our group obtained single-chain poly(methacrylic)-based nanoparticles *via* Cu^I-catalyzed [3+2] cycloaddition reaction between azide groups of a methacrylic copolymer backbone and alkyne groups of a homobifunctional cross-linker which consisted of a DTPA moiety.³⁸ The modulation of cross-linker equivalents and the dropwise addition of the polymer to a dilute cross-linker solution allowed the intramolecular collapse of random coils into SCPNs. It is important to note that most of the covalent strategies for the preparation of SCPNs suffer from the limitation of being performed at high temperatures, in the presence of metal catalysts and in organic solvents. In this context, collapsing polymer random coils into SCPNs in water media under mild conditions remains an unsolved issue. It is worth mentioning that metastable SCPNs have been recently prepared in water via host-guest interactions,^{39,40} but these examples are not based on covalent chemistry.

In order to develop a covalent-based synthesis of multifunctional SCPNs in water and mild conditions, we first designed and prepared the random co-polymer **8** (*tert*-butyl ester derivative precursor of **1**) (the formation of co-polymer **8** was followed by ¹H NMR, see Table S1) and the

homobifunctional linker **2** generated *in situ* from its parent 1,1-dimethoxy derivative **9** (see SI). Co-polymer **1** contains carboxylic and alkoxyamine groups, while linker **2** is based on a DTPA derivative that contains terminal aldehyde groups which have complementary reactivity with respect to the alkoxyamine groups (Scheme 1). The intra-chain collapse of the water-dispersible co-polymer **1** was carried out by the controlled addition of linker **2** in water at a controlled pH.

As mentioned above, our group previously obtained poly(methacrylic)-based SCPNs using a DTPA-based cross-linker which enabled chelation of Gd^{3+} cations for further MRI applications.³⁸ Inspired by this work, and considering that ^{67}Ga -radiolabeling was required before imaging studies, we decided to maintain the DTPA-based linker with slight modification in the terminal reactive groups (aldehyde functionalities instead of triple bonds).

Scheme 1. Schematic representation of the synthesis and functionalization of PMAAc SCPNs.

The formation of the PMAAc SCPNs was confirmed by different techniques including gel permeation chromatography (GPC) and transmission electron microscopy (TEM), nuclear magnetic resonance (NMR) and dynamic light scattering (DLS).

The appearance of the aldoxime signals at 6.8 ppm and 7.6 ppm in the ^1H NMR spectra confirmed the reaction of the oxyamine groups with the aldehyde of the linker (Figure 1). The presence of the linker was also evident from the new signals in the range 3-4 ppm (see also Figure S9). Picoline borane was selected to reduce residual aldehydes to hydroxyl groups and avoid side-reactions. The *O*-alkyl-oxime double bonds in the nanoparticles were not reduced by picoline borane in the reaction conditions as demonstrated by the above-mentioned ^1H NMR signals, in agreement with the literature.⁴¹

Figure 1. ^1H NMR in D_2O , 500MHz: A) Copolymer **1**; B) PMAAc SCPN (**3**).

GPC analysis gave an indication of single polymer chain collapse as a higher retention time for SCPN **3** when compared to the starting co-polymer **1** was found (Figure 2A). This result

indicates a lower apparent molecular weight of **3** with respect to **1**, despite the higher molecular weight of the former compound due to the presence of the cross-linker.

TEM analysis showed the presence of well dispersed particles (**Figure 2B**). The size of the collapsed nanoparticles obtained by TEM after spin-coating and negative staining was $d = 15 \pm 1$ nm. Aqueous cryo-TEM experiments confirmed the presence of well-defined nanoparticles (see Figure S13). Size distribution (average diameter) determined by TEM and cryo-TEM showed significant, but expected differences ($d = 15 \pm 1$ nm and 29 ± 7 nm, respectively). These differences are presumably due to the fact that cryo-TEM allows electron microscopy of aqueous (water-containing) specimen in their hydrated state, and it is well known that PMAAc are highly hydrated in water.

Figure 2. A) GPC chromatograms for co-polymer (*blue*) and the corresponding SCPNs (*green*). B) TEM image of PMAAc SCPNs.

The intensity-based DLS measurements in PBS buffer showed hydrodynamic diameters (Z-average) of ~ 45 nm (PDI 0.34). The relatively high polydispersity and the fact that DLS is very

sensitive to the scattering of large particles is the reason for the different results with respect to cryo-TEM.

After characterization, the SCPNs were further functionalized with targeting moieties. In spite of the literature debate on implication of SSTRs in pancreatic cancer, recent data showed that the somatostatin receptor subtype SSTR-2 is expressed in the majority of pancreatic tumors and could play a crucial role in patients with pancreatic adenocarcinoma.⁴² Hence, PTR86 was anticipated as an excellent candidate for conjugation to the NPs. Anchoring of the peptide to the surface of the SCPNs was favored by the presence of carboxylic acid moieties on the surface of the SCPNs which could react with the amino pendant group of lysine in FITC-PTR86. In fact, the functionalization of the SCPNs with PTR86 was achieved by coupling the amino-derivative of the peptide after activation of the carboxylic acid moieties of PMAAc SCPNs with DMTMM. In spite of the fact that the acid groups of the DTPA moieties are expected to be shielded inside the nanoparticles and their number is one order of magnitude less than the one of pendant acid groups in the copolymer, we cannot exclude that they do not react in the peptide-like coupling. However, the radiolabelling experiments (see below) demonstrated that the ⁶⁷Ga complex with DTPA or its eventual (mono)amidated analogue proved to be stable. The amount of peptide incorporated to the PMAAc SCPNs after conjugation and purification by exhaustive dialysis was found to be 7% w/w, as determined by fluorescent spectroscopy (see Figure S14). The DLS analysis showed a double distribution in case of PTR86-PMAAc SCPNs, probably due to some small aggregates, however no significant change in average size was observed (see Figure S12).

Cytotoxicity. Cell viability was evaluated *in vitro* after 72 hours of PMAAc SCPNs incubation with PANC-1 and five additional pancreatic cell lines (Figure 3). No significant reduction in cell viability was observed at concentration as high as 200 µg/mL in any of the cell lines analyzed.

Figure 3. *In vitro* cytotoxicity of PMAAc SCPNs (3). Results are represented as average values obtained from three independent MTT tests. Every single MTT test was performed in triplicate. The error bars in the graph represent standard errors (n = 3).

***In vivo* toxicity.** An *in vivo* acute toxicity screening that comprises clinical, hematological, clinical chemistry and pathological observations was used for preliminary toxicological assessment of the PMAAc SCPNs. Mice were treated with a starting dose of 100 mg/Kg since no reduction in cell viability was detected by using comparable concentration of *in vitro* MTT assay. With the 100 mg/Kg dose, a pro-coagulative effect that elicited thrombosis (**Figure 4**) at the inoculation site (tail vein) associated to peripheral consumption of platelets was constantly observed. One out of five mice treated with 12.5 mg/Kg had thrombosis of tail vein associated with a severe reduction of platelets by hematology studies (Figure S16). No systemic effects were observed in other tissues and organs. Clinical chemistry revealed moderate and sporadic increase of liver enzymes (GLDH, GLDH and ALT).

Figure 4. Histological picture of the severe thrombotic lesion at the inoculation site (tail vein) when high concentrated PMAAc SCPNs (100 mg/Kg) are administered (H&E, 100x).

As a main conclusion, 12.5 mg/Kg was the recommended maximum dose for *in vivo* experiments in order to avoid thrombosis (intravascular coagulation) of the tail vein (inoculation site) which was the most common adverse condition observed at higher doses. Moreover to avoid thrombosis at the inoculation site, it was recommended a slow intravenous inoculation of the SCPNs (30'') and the use of lower dose (as it is the case of SPECT experiments, see below).

Radiolabeling of SCPNs and free peptide and stability tests. Radiolabeling of SCPNs was performed by incubation of the nanoparticles with a solution of $^{67}\text{GaCl}_3$. The choice of the radioisotope was made taking into account the following factors: first, the half-life is sufficiently long to enable NP tracking during several days after administration; second, the gamma emission properties are very convenient for the subsequent visualization using SPECT imaging; finally, Gallium-67 is widely used in the clinical setting, and is commercially available at a reasonable cost. The presence of the DTPA derivatives as cross-linkers in SCPNs, previously applied to the

successful incorporation of Gd^{3+} , suggested the suitability of this radiometal to form stable metal-chelator complexes.³⁸

Labeling efficiency values were above 60% in all cases (Table 1) resulting in overall radiochemical yields after purification > 50%. Radiolabeled SCPNs **3** and **5** showed good radiochemical stability in physiological saline solution, with > 90% of the radioactivity attached to the SCPNs after 24 hours. These values were maintained at t=48 hours (Table 2). It is well known that chelators such as NOTA or DOTA form more stable complexes with ^{67}Ga than DTPA, and hence they could be anticipated as more convenient alternatives. However, further functionalization of the nanoparticles would be required to attach these chelators, with consequent increase in production-cost and NP complexity. As expected, the presence of DTPA at the core of the NPs proved to entrap the radiometal in a stable fashion, as demonstrated in stability studies. The stability of the radiolabeled NPs was sufficient to approach *in vivo* studies.

For the free peptide PTR86, NOTA was selected as the chelator of choice. High labeling efficiency was obtained when the incubation with ^{67}Ga was conducted at 70°C for 15 minutes. Incorporation ratios up to 78% were obtained, as determined from chromatographic profiles. After purification by solid phase extraction, free ^{67}Ga could be almost quantitatively removed from the final solution and only the presence of labeled peptide could be detected in the radiochromatogram. Non-decay corrected radiochemical yields above 50% were obtained (Table 1). The peptides proved to be relatively stable in physiological saline solution, with > 90% of the radiometal attached to the peptide after 3 hours of incubation. Progressive detachment of the radiometal was observed at longer times (Table 2).

Table 1. Radiolabeling efficiency and Radiochemical yield for SCPNs and free PTR86 peptide.

Labeled compound	Radiolabeling efficiency (%) ¹	Radiochemical yield (%) ¹	PTR86 loading (%)
SCPN 3	64 ± 5	54 ± 4	0%
SCPN 5	69 ± 2	57 ± 4	7%
PTR86-NOTA (6)	70 ± 4	52 ± 6	NA

¹Values are expressed as mean ± standard deviation (n=3)

Table 2. Stability of radiolabeled SCPNs and free PTR86 peptide, expressed as percentage of radioactivity attached to the NP, after incubation with saline solution at 37°C.

Labeled compound	2 min	1 h	3 h	24 h	48 h
SCPN 3	94 ± 4	93 ± 1	94 ± 4	95 ± 1	96 ± 1
SCPN 5	98 ± 9	98 ± 1	95 ± 5	97 ± 1	91 ± 7
PTR86-NOTA (6)	99 ± 1	96 ± 1	91 ± 3	81 ± 4	74 ± 6

***In vivo* imaging studies.** SPECT is an *in vivo*, non-invasive molecular imaging modality that provides information about the distribution of radioactive probes (labelled with a gamma emitter) within living organisms. Despite SPECT does not provide anatomical information, coregistration with CT images of the same animal are straightforward when hybrid imaging systems are used. In our case, sequential SPECT-CT imaging sessions of tumor bearing mice were carried out at 3, 24 and 48 hours after intravenous administration of the labeled SCPNs (**3** and **5**) or PTR86-NOTA (**6**).

For SCPNs, visual inspection of the images acquired at short times after administration (t=3h) revealed major accumulation of the nanoparticles in the liver, irrespective of the functionalization, suggesting high entrapment by the reticuloendothelial system (RES) organs (see Figure S17). Significant accumulation in the bladder was also observed, suggesting partial

degradation of the SCPNs or detachment of the radiolabel and elimination *via* urine. For the labeled peptide PTR86-NOTA, images acquired at short times after administration (t=3h) showed major accumulation in the kidneys and fast elimination via urine, with minor accumulation in the liver (Figure S17).

In order to assess the preferential accumulation of the targeted SCPNs in the tumor, volumes of interest (VOIs) were delineated in the tumor (T) and in the muscle (M) and T/M ratios were calculated. The same procedure was applied for imaging studies performed with the free peptide and non-targeted SCPNs (**Figure 5**).

Figure 5. Tumor-to-muscle (T/M) ratios at 3, 24 and 48 hours after administration of radiolabeled SCPNs and PTR86-NOTA. Columns: mean; bars: SD. Significant difference *P < 0.05, **P < 0.01.

At t=3 hours, very similar T/M values were obtained for the three labeled species, with values of 1.58 ± 0.23 , 1.76 ± 0.36 and 1.84 ± 0.27 for PMAAc SCPNs (**3**), PTR86-PMAAc SCPNs (**5**), and free peptide **6**, respectively. At t= 24 hours, T/M obtained for SCPNs **3** and **5** were

significantly higher than values obtained for the same labeled species at $t=3$ hours ($P = 0.0012$ and 0.0039 , respectively) suggesting a progressive accumulation of the SCPNs in the tumor due to EPR effect. Interestingly, T/M values for the targeted SCPNs **5** further increased after 48 hours, to reach a value of 5.16 ± 0.98 . The difference with respect to $t=24$ h was again statistically significant ($P = 0.026$). Of note, T/M values for non targeted SCPNs **3** did not increase in the period 24-48 hours. Altogether, these results suggest that SCPNs reach the tumor by EPR effect (this happens mainly at $t < 24$ hours). Once there, the presence of the targeting moieties improves retention within the tumor tissue, while clearance takes place in other organs or tissues in which SSTRs are not over-expressed, such as the muscle; these combined effects lead to a significant increase of the T/M ratio, which ultimately results in better tumor delineation on the images (see **Figure 6**).

Figure 6. SPECT-CT images of a mouse injected with PTR86-PMAAc SCPNs (**5**). Images were acquired 48 hours after administration of the radiolabeled NPs. *Left:* 3D-surface rendered CT image of the skeleton of the animal (grey scale) corregistered with a 3D-volume rendered PET image (in color); *Right:* selected coronal PET slices (in color) corregistered with the

corresponding CT slices (grey scale) for better visualization of the relative accumulation of radioactivity in major organs and tissues. Accumulation of radioactivity in the tumor (top, indicated with an arrow), the liver (middle) and bone junctions and bladder (bottom) can be clearly visualized.

Some of us recently reported on the use of recombinant human serum albumin (rHSA) loaded with magnetic γ -CAN maghemite NPs for medical imaging of pancreatic cancer,⁴³ studying the same tumor xenograft model used in the present paper. The ~150nm sized nanoparticles were covalently functionalized with a tissue plasminogen activator derived peptide (tPA) as targeting moiety and a DOTA-⁶⁷Ga complex. SPECT-CT analyses after intravenous administration indicated major accumulation in the liver and a T/M increase of approximately 2.50 from 3 h to 24 h (similar to our findings, see Figure 5). This result was also confirmed by conventional T1-weighted magnetic resonance imaging. However, after 48 hours T/M did not further increase and no significant difference between tPA-targeted and non-targeted NPs was observed, unlike our PTR86-targeted SCPNs which reached a T/M value above 5. This result may be due to the nature of the nanoparticles and/or to the targeting efficiency of the peptide moiety. Using a different PANC-1 bearing mouse model, Amiji et al. evaluated the biodistribution of PEG-modified and epidermal growth factor receptor (EGFR)-targeted thiolated type B gelatin nanoparticles after intravenous administration.⁴⁴ Fluorescence imaging after encapsulation of the near-IR dye indocyanine green showed quick uptake (1 h) and sustainable accumulation (up to 12 h) in tumor of both targeted and non-targeted NPs. At early time points, ¹¹¹In-labelled EGFR-targeted NPs showed two-fold accumulation in the tumor respect to non-functionalized NPs, as observed by quantitative gamma counter analysis of the organs *ex vivo*. On the contrary, at late time-points the difference was not so striking. These examples also highlight the importance of having at

disposal different imaging modalities to face the challenge of early detection pancreatic tumor¹⁸ and renewed efforts are being devoted also to facilitate accumulation nanoparticulate-based contrast agents in the pancreas,⁴⁵ as imaging of pancreatic tissue is particularly difficult.

CONCLUSION

We have presented a multifunctional nanoparticle platform that combines imaging capability and receptor specificity for pancreatic cancer detection and that is based on water dispersible poly(methacrylic acid) (PMAAc)-based single-chain polymer nanoparticles (SCPNs). Simultaneous incorporation of the targeting peptide PTR86 and the radionuclide ⁶⁷Ga for SPECT imaging was achieved. SCPNs showed interesting stability, low toxicity, biocompatibility and *in vivo* targeting properties towards a subcutaneous pancreatic adenocarcinoma model in mice. The results obtained in this study indicate higher retention of the nanoparticles in the tumor when these were decorated with the PTR86 peptide, leading to higher tumor-to-muscle ratios as determined from *in vivo* images. These nanoparticles seem promising for further development, and ongoing experiments are demonstrating that they do not induce pro-metastatic side effects. Incorporation of anti-cancer drugs is currently underway to combine the diagnostic characteristics of the SCPNs with a therapeutic effect. This *in vivo* proof of concept for pancreatic cancer may serve as a starting point for further applications of functionalized SCPNs in different solid cancer diagnostics and, in perspective, nanotherapies.

ASSOCIATED CONTENT

Supporting Information. Synthesis, purification and characterization of PTR86 peptide derivatives and SCPNs. Graphs of MTT tests for *in vitro* toxicity and selected images of

hematology studies. Coronal SPECT projections of tumo-bearing mice at t=3 hours after administration of ^{67}Ga -labelled compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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