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Implantable controlled release devices for BMP-7 delivery and suppression of glioblastoma initiating cells

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ABSTRACT

Designing therapeutic devices capable of manipulating glioblastoma initiating cells (GICs) is critical to stop tumor recurrence and its associated mortality. Previous studies have indicated that bone morphogenetic protein-7 (BMP-7) acts as an endogenous suppressor of GICs, and thus, it could become a treatment for this cancer. In this work, we engineer an implantable microsphere system optimized for the controlled release of BMP-7 as a bioinspired therapeutic device against GICs. This microsphere delivery system is based on the formation of a heparin-BMP-7 nanocomplex, first coated with Tetronic® and further entrapped in a biodegradable polyester matrix. The obtained microspheres can efficiently encapsulate BMP-7, and release it in a controlled manner with minimum burst effect for over two months while maintaining protein bioactivity. Released BMP-7 showed a remarkable capacity to stop tumor formation in a GICs cell culture model, an effect that could be mediated by forced reprogramming of tumorigenic cells towards a non-tumorigenic astroglial lineage.

Keywords: cancer initiating cells, glioblastoma, controlled release, bone morphogenetic protein, microspheres, PLGA.

1. INTRODUCTION

High-grade malignant astrocytic gliomas are the most common and aggressive primary central nervous system tumors, accounting for 52% of all primary brain cancer cases [1]. Glioblastoma multiforme (GBM) is the most common glioma in adults and the typical survival of the patients is around one year [2, 3], a prognosis that has stalled in the last decades. It is now becoming clear that this intrinsic resistance of GBM to current treatments is caused by a cell subpopulation with high resistance to radiation and chemotherapy: glioblastoma initiating cells (GICs) [4-7]. GICs are also responsible for tumor reinitiation and sustained growth and this is why they are conceptualized as cancer´s locomotive engine [8]. Besides, the location of glioblastomas in the brain greatly restricts chemotherapeutic options. For illustration, it is considered that over 98% of small molecular weight, and essentially 100% of high molecular weight drugs are not transported across the blood-brain-barrier [9, 10].

Recently, Chirasani *et al.* disclosed a new physiological role for endogenous neural stem cells. This work showed that these stem cells secrete bone morphogenetic protein-7 (BMP-7), and that this growth factor acts as a paracrine suppressor of GICs. Animal experiments also showed that these cells will migrate to the borders of neoplastic foci to suppress GBM formation, an antitumoral protective mechanism that could be expected to diminish with age as the neural stem cell pool is exhausted [11]. Indeed, this and other studies have shown that BMPs can arrest cell cycle in glioblastoma cells [12], and suppress the tumorigenic capacity of GICs by inducing their differentiation to phenotypes with lower levels of stem cell markers [11, 13, 14]. There is now a body of evidence supporting a role of BMP-7 as a reprogramming factor of GICs, a process that should remove the tumors capacity for recurrence.

We propose the use of BMP-7-loaded biodegradable implantable microspheres as bioinspired therapeutic strategy against GICs. The clinical concept would be to implant these controlled release devices upon surgical resection of the primary tumor, to prevent GBM reinitiation. This approach intends to overcome two critical constraints for successful delivery of BMP-7 to the brain: (i) its inability to cross the blood-brain-barrier, and (ii) its short physiological half-life (few minutes [15]). Several intracranial implantable devices have already been described for the treatment of GBM with hydrophobic anticancer drugs [8, 16], and one of them is already marketed (Gliadel® wafer). The delivery system proposed herein is nevertheless particularly designed for the

encapsulation of a labile hydrophilic morphogen and for providing sustained release properties over several weeks while preserving the bioactive conformation of the protein, often a critical problem for encapsulated biopharmaceuticals [17].

Our strategy was to develop a delivery platform by a nanocomplex encapsulation technique. This method is based on the preparation of a nanocomplex from the specific binding of morphogens to heparin [18], followed by coating this nanocomplex with a cationic polyoxyethylene oxide derivative (Tetronic-coated nanocomplex). The resulting Tetronic-coated nanocomplex is compatible with biodegradable controlled release polymers [19, 20], and could be easily entrapped in poly(lactide-co-glycolide) (PLGA) microspheres. In this manuscript we report the design of this delivery platform, the physicochemical, and pharmaceutical characteristics of the resulting microspheres.

2. MATERIALS AND METHODS

2.1 Materials

For cell studies, we used an immortalized human glioblastoma cell line U-87MG (ATCC HTB-14™), with an intact BMPs signaling pathway and expressing low levels of BMP-7 [12]. Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle's Medium (DMEM)/ Nutrient F-12 Ham (DMEM/F12 1:1), B-27 serum-free supplement, L-glutamine, penicillin-streptomycin, trypsin and fetal bovine serum were purchased from Invitrogen (Spain). Accumax™ was obtained from Millipore (Spain). Propidium iodide was purchased from Calbiochem (Spain). BD Cytofix™ Fixation Buffer, BD Phosflow™ Perm Buffer III, anti-human Ig PerCP-Cy™5.5-labeled anti-nestin, Ig phycoerythrin (PE)-labeled anti-doublecortin and Ig PE-labeled anti-glial fibrillary acidic protein (GFAP) were purchased from BD Biosciences (Spain). Poly(D,L-lactide-co-glycolide) 50:50 Resomer® RG 503 (PLGA) (MW=34 kDa) was purchased from Boehringer Ingelheim (Germany). Tetronic® 1107 (T1107, HBL=24 MW=15 kDa), α-chymotrypsinogen A from bovine pancreas (pI 9.5 MW=25.7 kDa), heparin sodium salt grade IA from porcine intestinal mucosae, cotton seed oil, soybean lecithin, bromodeoxyuridine (BrdU), fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody, Ribonuclease A (RNAse) from bovine pancreas, Triton X-100, avidin-peroxidase conjugate and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Aldrich (Spain). Recombinant human epidermal growth factor (EGF), recombinant human fibroblastic growth factor-basic (bFGF), recombinant human bone morphogenetic protein 7 (BMP-7) (pI 8.1 MW=28.8 kDa), polyclonal antibody rabbit anti-human BMP-7 and polyclonal antibody biotinylated rabbit anti-human BMP-7 were purchased from PeproTech (UK). All other solvents and chemicals used were of high-grade purity.

2.2. Microsphere preparation

The procedure for preparation of the microspheres was based on previous methods described by our group [21, 22], but conveniently modified for the efficient encapsulation of heparin-binding proteins (nanocomplex encapsulation technique). For optimization of the methodology, we first encapsulated α-chymotrypsinogen as a model protein. Briefly, 20 μg or 40 μg of α-

chymotrypsinogen (for theoretical loadings of 0.1 and 0.2% w/w, respectively) and heparin (1:1 mass ratio with the protein) were dissolved in 300 µL of sterile water and allowed to interact for 30 minutes at room temperature (RT). Then, 2.5 mg of Tetronic T1107 were added to this solution and the mixture was allowed to interact for other 30 minutes to form Tetronic-coated nanocomplexes. These Tetronic-coated nanocomplexes were directly freezed-dried without adding any cryoprotectant to consolidate molecular interactions (primary drying at -35°C, secondary drying at 25°C; VirTis Liophylizer, Genesis 25ES, USA). The freeze-dried cake was resuspended in 400 µL of acetonitrile containing 20 mg of PLGA for a final PLGA:T1107 8:1 mass ratio. The organic phase was then added dropwise to 4 mL of cotton seed oil containing 0.5% (w/v) soybean lecithin. After that, the resulting suspension was sonicated for 20 seconds (output 40W, Branson Sonifier 250, Danbury, CT, USA) and stirred for 45 min in an extraction hood. Two milliliters of petroleum ether were added to the suspension to harden immature microspheres, and the preparation was agitated for another 10 minutes in the extraction hood. Finally, microspheres were collected by filtration under vacuum using a nitrocellulose membrane (25 mm, 0.22 µm). Collected microspheres were washed with petroleum ether, freeze-dried and stored at 4°C until use.

The same procedure was used to encapsulate BMP-7. In this case, 2 µg of BMP-7 (theoretical loading of 0.01% w/w) and 2 µg of heparin (i.e. 1:1 mass ratio) were dissolved in 300 µL of sterile water. Then, these nanocomplexes were processed as described before.

2.3. Morphology and size characterization of the microspheres

Particle size and morphology of the microspheres were analyzed by scanning electron microscopy (SEM) (LEO-435vp, UK). Samples were stuck on metal stubs and coated with gold-palladium under vacuum. The size of the microspheres was calculated from micrographs using the computer program Digital Micrograph™ 3.7.0 (Gatan Software, USA).

2.4. Determination of the encapsulation efficiency for α-chymotrypsinogen

The encapsulation efficiency of α-chymotrypsinogen was evaluated following a modification of a previously described protein extraction method [23]. Briefly, 5 mg of microspheres were dissolved in 100 µL of dimethyl sulfoxide under continuous agitation (350 rpm, Heidolf, Promax 2020, Germany) during 1 hour. Then, 400 µL of 0.05M NaOH solution containing 0.5% (w/v) SDS were

added to the microspheres dissolution and the mixture was incubated for another hour under the same conditions. The resulting sample was centrifuged at 7000 RCF during 5 minutes at 25ºC. The extracted α-chymotrypsinogen was quantified in the supernatant by a standardized bicinchoninic acid assay (Micro BCA protein Assay Kit Pierce Biotechnology Inc., USA).

2.5. Determination of the encapsulation efficiency for BMP-7

The amount of BMP-7 encapsulated in the microspheres was directly quantified by a protein extraction method. Briefly, 1 mg of the microspheres was dissolved with 1 mL of dichloromethane under continuous agitation (350 rpm, Heidolf, Promax 2020, Germany) for 1 hour. The solution was filtered under vacuum using a nitrocellulose membrane (25 mm, 0.22 µm). Then, the nitrocellulose membrane was incubated in 3 mL of PBS (pH 7.4) containing 0.1% (w/v) BSA and 0.05% Tween 20. The membrane was incubated for 6 hours under mild agitation (Heidolph, Promax 2020, Germany) at 250 rpm. The extracted BMP-7 was quantified by an enzyme-linked immunosorbent assay (ELISA) as described below (section 2.7.).

2.6. *In vitro* release studies

Samples comprising 1 mg of PLGA:T1107 (8:1 mass ratio) loaded with BMP-7 and heparin (and controls without BMP-7) were incubated with 500 µL of (i) PBS (pH 7.4) containing 1% (w/v) BSA or (ii) mitogen-free neurospheres (NSs) culture medium, under agitation (100 rpm) and at 37ºC. At several scheduled time points, from 12 hours to 90 days, microspheres were centrifuged at 7000 RCF for 10 min at 4ºC, and supernatants collected. Antigenically active BMP-7, released from the microspheres to the supernatants, was quantified by ELISA as described in the next section.

2.7. BMP-7 quantification by ELISA

A 96-well microtiter plate was coated with 100 µL of polyclonal antibody rabbit anti-human BMP-7 (1 µg/mL in Dulbecco's PBS, overnight incubation at 4ºC in a humid container). The plate was washed three times with PBS containing 0.05% Tween 20 (PBST). To minimize unspecific interactions, 300 µL of PBS containing 1% (w/v) BSA were added to each well and the plate was incubated for 1 hour at 37ºC in a humid container. After this time, the plate was washed three times with PBST and 100 µL of BMP-7 standard solution and test samples were added to the

wells. All standards and test samples were always conveniently diluted in PBS containing 0.1% (w/v) BSA and 0.05% Tween 20 (PBST-BSA). The plate was incubated for 4 hours at 37ºC in a humid container and washed thoroughly. Afterwards, 100 µL of polyclonal biotinylated rabbit anti-human BMP-7 (0.5 µg/mL) in PBST-BSA were added to each well. The plate was incubated for 2 hours at 37ºC in a humid container and washed three times with PBST. Afterwards, 100 µL of avidin-peroxidase conjugate diluted 1:2000 in PBST-BSA were added to each well and the plate was incubated for 1 hour at 37ºC in a humid container. The plate was washed three times and 100 µL of substrate (ABTS) added to the wells. After color development, the plate was read at 405 nm on a microplate reader (Biorad, Microplate reader model 680, Japan).

2.8 U-87MG neurosphere culture

Immortalized U-87MG cells were grown in DMEM medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum, in a 5% CO_2, 95% humidity incubator. Cells were passaged 1-2 times a week. U-87MG cells were then cultured as NSs, being seeded in NSs formation medium (DMEM/F-12 (1:1), 1% L-glutamine, B-27 serum-free supplement 1X, 1% penicillin-streptomycin, 20 ng/mL EGF, 20 ng/mL bFGF). For expansion of the culture, after 6 days, NSs in suspension were collected by centrifugation, disaggregated with Accumax™ and re-seeded at a density of 40,000 cells/mL in fresh NSs culture medium in a 100 mm culture plate. Prior to any experimentation, cells were dedifferentiated under these conditions for 6 weeks.

2.9. Neurosphere formation assays

NSs were disaggregated and individual U-87MG cells plated at clonal conditions (500 cells/well, in a 96-well culture plate, 200 µL of NSs growing medium). The effect of BMP-7 was tested by adding soluble BMP-7 (in PBS containing 1% (w/v) BSA) to the control medium (50 ng/mL final concentration per well). Neuropheres were allowed to grow for 6 days, and their number and size were determined by imaging with an optical microscope (Olympus 1X7) with a coupled camera (Olympus DP50), and using the software Cell A version 2.6 (Olympus Soft Imaging Solutions GmbH).

Subsequent experiments involved the collection of BMP-7, released from BMP-7-loaded microspheres incubated in NSs medium, at days 30 and 90. The final concentration of released BMP-7 was adjusted to 50 ng/mL (dilution with fresh culture medium). These samples were then tested in the NSs formation assays as described above. To consider the potential effect of other components that could be released from the microspheres (due for example to matrix degradation), other controls were additionally tested, namely: (i) 50 ng/mL of heparin; (ii) 313 μg/mL of Tetronic T1107; (iii) a mixture of BMP-7, Tetronic T1107 and heparin at the concentrations described before; (iv) release medium from blank microspheres at 30 and 90 days; (v) control medium incubated at 37ºC for 30 days.

2.10. Cell cycle/BrdU incorporation studies

U-87MG were cultured in NSs medium either with or without BMP-7 (50 ng/mL) for 6 days. NSs were then pulsed with BrdU (100 μM, 8 hours incubation at 37ºC). After this time, NSs were acutely disaggregated in single cell suspension and washed with PBS. For fixation, ice-cold ethanol was added to 1 million cells to a final concentration of 75% (v/v). For BrdU antibody staining, cells were washed with PBS, incubated first with 2N HCl in PBS (20 minutes at RT), and then with 0.1M sodium borate solution in PBS pH 8.5 (2 hours at RT). Finally, cells were washed and incubated with 10μL of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (1:10 dilution in PBS containing 0.5% (w/v) BSA and 0.5% Tween 20), 1 hour at RT. Cells were also stained with propidium iodide (10 μg/mL in PBS, with 500 μg/mL RNAse and 0.1% Triton X-100, overnight incubation at 4ºC) to determine total DNA content. Cells were analyzed by Fluorescence Activated Cells Sorting (FACS) (Becton Dickinson, model FACScan™, Spain).

2.11. Immunocytochemistry

To determine potential differentiation of U-87MG NSs (stem-like cells) upon incubation with BMP-7, specific surface cell markers for neural stem cells, neurons and/or astrocytes, were examined by FACS. U-87MG cells from disaggregated NSs, exposed or not to BMP-7, were washed with PBS and centrifuged (5 min, 850 RCF). The pellet was resuspended with BD Cytofix™ Fixation Buffer and incubated for 20 minutes at RT in the dark. Then, cells were permeabilized for 30 minutes with ice-cold BD Phosflow™ Perm Buffer III, also in the dark. Cells were washed twice and incubated

on ice for 30 minutes in the dark with 100 µL of PBS containing 0.5% (w/v) BSA and 5 µL/test of each conjugated antibody for 1 million of cells (anti-human Ig PerCP-Cy^{TM}5.5-labeled anti-nestin, Ig PE-labeled anti-doublecortin, Ig PE-labeled anti-GFAP). The cells were washed again thoroughly with PBS and resuspended in 400 µL of this buffer for flow cytometry analysis (Becton Dickinson, FACScan^{TM}, Spain).

2.12. Statistical analysis

The size data of microspheres and NSs were analyzed by a one-way ANOVA and a multiple comparisons test of Tukey as post-hoc ($p<0.05$). All the data were analyzed using the software Statistica 6.0.

3. RESULTS

3.1. Design of polymeric devices for controlled protein delivery

3.1.1 Microsphere preparation and encapsulation of α-chymotrypsinogen

Microspheres were prepared by a newly designed procedure optimized for growth factor encapsulation we termed the nanocomplex encapsulation technique. In this method, the protein is sequentially complexed by two protective excipients, heparin and Tetronic. Afterwards, these Tetronic-coated nanocomplexes are freeze-dried and the resulting polymer cake suspended in a PLGA organic solution. PLGA microspheres can then be casted from this organic phase through standard procedures, in this case by an O/O emulsion-solvent evaporation technique (for details see methods and Fig. 1).

We selected α-chymotrypsinogen as a model protein to optimize microsphere preparation and protein encapsulation due to its similar molecular weight and isoelectric point to BMP-7. Microspheres prepared by the nanocomplex encapsulation technique displayed smooth surface and regular spherical shape (Fig. 2, A-B). The particle size distribution was broad, typical of emulsification methods [24], and had mean values between 10 and 30 μm (Table 1). The production yield was excellent for all preparations, above 80%. This feature together with the reported physicochemical data suggested the suitability of the production method.

Microspheres prepared by the nanocomplex encapsulation technique showed a remarkable capacity to encapsulate the model protein. The encapsulation efficiency of α-chymotrypsinogen in the microspheres was, upon consideration of the statistical deviations, close to 100%; this value was independent of the theoretical protein loading for the two levels tested, i.e. 0.1 and 0.2%. For benchmarking purposes, we also studied the properties of microspheres prepared by a W/O/W double emulsion-solvent evaporation technique, the gold standard method for protein encapsulation in PLGA particles (see Supplementary Methods). Microspheres prepared by the double emulsion method presented similar characteristics than those from the nanocomplex encapsulation technique regarding particle size, particle size heterogeneity, morphology and production yield (Table S1, Fig. S1). Their encapsulation efficiency, however, was much lower: 30-40% (Table S1).

3.1.2 BMP-7-loaded microspheres

Encouraged by the promising results obtained for α-chymotrypsinogen, we applied the microspheres prepared by the nanocomplex encapsulation technique to BMP-7 delivery. The characteristics of BMP-7-loaded microspheres were similar to those observed for the α-chymotrypsinogen formulations (Table 2; Fig. 2, C-D), and BMP-7 encapsulation was also very efficient, above 90%. We consider this last result to be particularly remarkable since encapsulation data were obtained from direct determinations, based on protein extraction procedures and quantified by ELISA. Therefore, some protein might have not been correctly extracted from the matrix, or could have been rendered antigenically inactive during the extraction procedure, making the value determined a minimum estimate.

The *in vitro* release profiles of BMP-7-loaded microspheres were studied both in PBS (pH 7.4) containing 1% w/v BSA, and in mitogen-free NSs culture medium at 37ºC (Fig. 3). The release kinetics in both media follows a triphasic profile typical of PLGA microspheres: burst effect, the lag phase and the sustained release phase [19, 21]. For these microspheres the burst effect was negligible, below 3% of the protein payload. This absence of burst suggests that the amount of BMP-7 on the particle surface is minor, and it is a positive feature of the technology since uncontrolled burst release is commonly one order of magnitude larger for this kind of formulations [19, 22, 24, 25]. The lag phase, attributed to the time necessary for generating channels in the PLGA matrix amenable for proteins to percolate lasted for 15 days in PBS-BSA, and was a little shorter in mitogen-free NSs culture medium. From this point, the microspheres showed a sustained release of BMP-7 until the 60 days time-point.

We observed that almost 80% of the BMP-7 microsphere payload is released in its antigenically active conformation after 90 days in mitogen-free NSs medium (Fig. 3). This result is critical since it shows that the selected microsphere preparation with the Tetronic-coated nanocomplexes is capable of protecting BMP-7 during the release phase.

3.2 *In vitro* antitumoral effect in a glioblastoma initiating cell model

Having observed a sustained release of antigenically active BMP-7 from the microspheres, we decided to study whether the released morphogen had also preserved its bioactivity, and to evaluate its efficacy as a tumor suppressor in a GICs model. Previous studies by Piccirillo et al.

have shown that the glioma cell line U-87MG cultured in standard 2D conditions is insensitive to BMPs, a behavior not replicated by primary cultures of GICs [14]. In this work, we restored U-87MG cells sensitivity to BMPs by inducing their dedifferentiation by cultivation in the form of NSs [26]. We confirmed by FACS analysis that U-87MG cells cultured as NSs present a highly undifferentiated phenotype: almost all cells (>90%) were nestin positive (a neural stem cell marker), while only about 20% of them were positive for GFAP or doublecortin (astroglial and neural differentiation markers, respectively) (Fig. S2).

For NS formation assays, U-87MG cells were seeded at clonal conditions, a setup where each NS grows from one initiating cell. Due to this, the clonogenic index of the culture is a direct measurement of the fraction of cells behaving as GICs. We observed that under control conditions around one half of the seeded cells would produce a NS; we refer all clonogenic indexes reported to this value (i.e. clonogenic index of the control=1).

In a first study, we confirmed that BMP-7 has a tumor suppressive effect in the NSs formation assay, and that a suitable response was achieved for a dose of 50 ng/mL. This concentration was hence fixed for the rest of the experiments.

At a second stage, we aimed to test the potential effect of the different microsphere components that could be leached out during the release studies in the NSs formation assay (Fig. 4A). Tetronic showed a significant but minimal reduction in the clonogenic index and heparin had no effect on this parameter. BMP-7 led to a 30% reduction in the clonogenic index and this value was not modified by the presence of heparin and Tetronic (Fig. 4A, “Mixture”).

We also analyzed how the different components could have an impact on the size of the resulting NSs (Fig. 4B). Addition of BMP-7 resulted in a slight increase in the NSs size. Surprisingly, Tetronic and particularly heparin induced some reduction in the NSs size. The experimental group having BMP-7, Tetronic and heparin (“Mixture”) showed similar NSs size as compared to the control, probably due to a neutralization of the effects observed for BMP-7 and heparin/Tetronic.

At a third stage, we tested the effect of BMP-7 released from the microspheres and the proper controls in the NSs formation assay (Fig. 4, C-D). For that, we took samples of BMP-7 released from the microspheres in NSs culture medium (henceforth "release medium"), and we adjusted morphogen concentration to 50 ng/mL. BMP-7 released from the microspheres at 30 and 90 days

induced a dramatic reduction in the clonogenic index (Fig. 4C, 30dR and 90dR, respectively). This result was surprising since BMP-7 in solution at 50 ng/mL was less effective (30%). To explain this difference, we tested the effect of release medium previously incubated with blank microspheres for 30 and 90 days (30dC and 90dC, respectively). The results showed that this release medium generates by itself an inhibitory effect on NS formation, probably due to consumption of some nutrients from the media during microsphere incubation. This hypothesis is further clarified since we observed similar effects from release medium incubated at 37ºC for extended periods of time, even in the absence of microspheres (data not shown).

Despite the background noise arising from the suppressive action of the incubated release medium, BMP-7 bioactivity could still be detected in this assay: a physical mixture of release medium incubated with blank microspheres for 90 days and BMP-7 (50 ng/mL) led to further reductions in NS formation (Fig. 4C, 90dC+BMP7) as compared to the same conditions without BMP-7. According to the statistical analysis, BMP-7 released from the microspheres resulted in a further significant decrease in the clonogenic index as compared to this physical mixture of BMP-7 and 90-days control release medium ($p<0.05$).

BMP-7 released from the microspheres also drastically reduced NSs size (90dR, Fig. 4D). We explain this reduction by the simultaneous effect of the other released components (i.e. Tetronic and heparin, see Fig. 4B), and also by the effect of the consumption of the cell culture medium during incubation, as discussed above. Both of these factors were quantified by measuring NSs size for release medium incubated with blank microspheres for 90 days (90dC, Fig. 4D). Again, it is interesting to note that BMP-7 released from the microspheres was slightly more effective reducing NSs size as compared to a physical mixture of BMP-7 and microsphere-incubated release medium (90dC+BMP7, Fig. 4D).

The effect of BMP-7 in the NSs culture is illustrated through microscopy images in Fig. 5. BMP-7 in solution does not generate smaller NSs, but rather a lower number of them as compared to the control. Release medium incubated with blank microspheres, on the other hand, did have an effect in NSs size as mentioned above. Addition of BMP-7 to release medium incubated with blank microspheres had a combined effect as NSs were small and there was a higher fraction of cells that never initiated NSs formation (arrows). BMP-7 released from the microspheres had the largest effect of all, both on NSs size and NSs suppression.

3.3 Mechanistic studies on BMP-7 effect

Based on the biological effects observed, we performed some preliminary experiments to define the mechanism of action of BMP-7 in the U-87MG NS model. A limitation of this model is that BMP-7 actions seem to be mainly restricted to the tumor initiation stage, whereas only after NSs had growth we were able to collect enough cells to run proliferation and phenotyping assays. Therefore, we take these results with caution and we advise to consider them within the context of other studies performed with primary cultures.

We first studied BMP-7 effect on cell cycle and proliferation by using a BrdU incorporation assay. We noted that BMP-7 supplementation does not result in changes in the cell cycle profile, nor in BrdU incorporation (Fig. 6). This suggests that BMP-7 does not exert long-lasting effects on proliferation, a conclusion that is supported by our previous observation regarding NSs size upon culture with BMP-7 (Fig. 3B).

An alternative hypothesis to explain why a fraction of the cells never started proliferation even though BMP-7 has no long-lasting effect on the cell cycle is that it forces differentiation of the cells towards a non-dividing lineage; this implies a permanent loss of their GIC phenotype. Our results regarding this hypothesis were not totally conclusive: on one hand, the stem cell marker nestin was not reduced for the cell population upon culture with BMP-7. However, we did see some small phenotype changes in BMP-7 treated cells, which were less doublecortin positive and more GFAP positive (Fig. S2). This effect agrees with previous results from primary culture studies suggesting shifting of GICs towards the astroglial lineage. In our experiment, some of the potential pro-differentiation effect of BMP-7 might be masked by the stated limitations of the model, as well as by the dedifferentiation effect of the NSs culture medium used that contains EGF and bFGF.

4. DISCUSSION

Dismaying clinical outcomes for glioblastoma treatment seem to be linked to the presence of a chemotherapy-resistant cell population with capacity to reinitiate tumor growth: glioblastoma initiating cells (GICs) [4, 27]. Academic scientist, and recently pharmaceutical industry, have reacted to this paradigm shift by actively seeking drugs capable of targeting cells with tumor initiating capacities [28]. BMPs have shown GICs suppressor capacity, an effect that seems to be mediated by reprogramming of GICs towards terminally differentiated phenotypes [13, 14, 29].

We postulate that the therapeutic efficacy of BMPs *in vivo* will be compromised if they are not integrated in a suitable carrier capable of enhancing their biological half-life and deliver them to their target region in the brain. This postulate is supported by the fact that clinically-approved medicines including BMPs all have in common the use of a delivery platform [30]. Our therapeutic proposal is that we can suppress GBM recurrence through the use of a BMP-7-loaded controlled release device delivered to the brain by implantation upon primary tumor resection. For that, we aimed to encapsulate BMP-7 in microspheres suitable for implantation by minimally-invasive surgery and sporting controlled release properties. By providing sustained BMP-7 levels at the tumor site, the microspheres would mimic a physiological protective mechanism: in young individuals, neural stem cells are recruited to neoplastic foci where they deliver BMP-7 as a paracrine tumor suppressive agent [11].

We selected PLGA as the microspheres' biodegradable polymer owing to its favorable regulatory status for implantation, and to previous positive experiences for the intracraneal delivery of cytotoxics [16, 31-33]. Although PLGA devices have been widely investigated for the controlled release of biopharmaceuticals [34], labile drugs can be degraded under release conditions when encapsulated in these materials due to a known internal acidification process resulting from polymer degradation [17, 19]. In the present work, BMP-7 was encapsulated together with heparin and Tetronic as protective excipients, a similar technology to that previously reported by our group to encapsulate plasmid DNA, fibroblast growth factor, platelet derived growth factor and other peptidomimetics [19-22, 25, 35]. Herein, however, we enhanced the interaction of BMP-7 with the protective excipients by selecting materials capable of specifically interacting with the drug (BMP-7-heparin) or capable of interacting ionically with the BMP-7-heparin nanocomplex

(Tetronic). The interaction among the components of the Tetronic-coated nanocomplexes was then consolidated by freeze-drying. This method resulted in compositions showing almost complete encapsulation of BMP-7, absence of burst release, and sustained protein release for over two months in its antigenically active conformation. We believe that this efficient encapsulation of the morphogen results from its integration in Tetronic-coated nanocomplexes that should have higher interpolymer compatibility *vis a vis* with the PLGA matrix.

The efficacy of the microsphere formulation was tested in the U-87MG cell line, cultured as NSs to induce GIC-like properties. NS models from immortalized cell lines present limitations compared to primary cultures of GICs, most of which have been outlined before in this manuscript. However, they also present relevant advantages: (i) they are readily available and do not raise particular ethical issues; (ii) they are accessible for any laboratory in unlimited quantities, which is ideal for drug screening; and finally, (iii) they should lead to higher comparability of results among different laboratories [36]. Our studies have confirmed the tumor suppressive effect of BMP-7 in the reported NSs model, and we have discarded the possibility that this effect might be mediated by an antiproliferative mechanism. Although we have not been able to confirm this end, it is more likely that the tumor suppressive effect is mediated by directed differentiation of GICs towards the astroglial lineage as previously reported [11].

Using the U-87MG NSs model, we have confirmed preserved bioactivity of the BMP-7 released from our microspheres, even from samples obtained at the 90 days release point. Interestingly, the effect of BMP-7 in NSs model was even greater for the protein released from the microspheres than for a physical mixture of soluble BMP-7 and the blank microspheres release medium. With these results it is tempting to speculate if heparin, Tetronic and BMP-7 might be released from the microspheres in a structured form (i.e. the Tetronic-coated nanocomplex) that could increase their tumor suppressive effect. In this sense, heparin is known to modulate the pharmacological effect of several binding proteins [37], and several works also report genome-wide effects for Tetronics and other polyethylene glycol derivatives [38]. Future studies of our group would try to clarify this point.

This work is, to the best of our knowledge, one of the pioneers showing a biomaterials-based reprogramming strategy for cancer treatment, and to propose biodegradable implantable devices for the controlled release of BMPs as a potential GICs suppressive treatment in glioblastoma. We have also reported a potential technological platform for this application, showing suitable

characteristics for drug encapsulation, controlled release and pharmacological efficacy in a relevant cell model.

5. CONCLUSIONS

Herein we report a controlled release device loaded with a BMP as a potential medicine against cancer initiating cells in glioblastoma treatment. To achieve suitable delivery of the BMP in the intracranial cavity, we designed a technological platform where BMP-7 was incorporated into biodegradable microspheres after enforced interaction with two protective excipients: heparin and Tetronic. The delivery systems prepared by this method presented remarkable characteristics: almost complete encapsulation, absence of burst release and sustained release of BMP-7 for at least two months in a bioactive form. The BMP-7 released from the microspheres was capable of suppressing tumor initiation in a glioma initiating cell-like culture model.

6. AUTHOR CONTRIBUTIONS

ERN, NC and MGF conceived the study. All authors designed experiments. ERN and CR performed experiments. All authors analyzed data and wrote the manuscript.

7. CONFLICTS OF INTEREST DISCLOSURE

ERN, NC and MGF are co-inventors of the patent application PCT/ES2013/070655, related to the work being published. As co-inventors we might be subject to financial compensation in case of future patent exploitation. Besides that we declare no other conflicts of interest.

8. ACKNOWLEDGEMENTS

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Tables

Table 1. Physicochemical and pharmaceutical characteristics of microspheres obtained by the nanocomplex encapsulation method loaded with α-chymotrypsinogen (0.1 and 0.2% w/w theoretical loading). The composition of the microspheres comprised poly(lactic-co-glycolic acid) (PLGA), Tetronic (TR) and heparin (HP). The studied characteristics were: production yield, particle size and encapsulation efficiency (EE). Results are means ± SD, n=3.

Formulation	Theoretical loading (%)	Yield (%)	Size (μm)	EE (%)
PLGA	_	84.4 ± 8.2	27.9 ± 11.8	-
PLGA:TR	_	79.6 ± 6.6	22.0 ± 9.8	-
PLGA:TR:HP*	_	87.7 ± 6.2	9.0 ± 4.2	-
PLGA:TR:HP	0.1%	83.3 ± 2.9	22.9 ± 10.3	98.5 ± 1.6
PLGA:TR:HP**	_	86.1 ± 3.9	9.7 ± 4.2	-
PLGA:TR:HP	0.2%	87.2 ± 7.7	9.2 ± 2.5	105.5 ± 9.7

* Control with a heparin amount corresponding to the 0.1% loading formulation.

** Control with a heparin amount corresponding to the 0.2% loading formulation.

Table 2. Physicochemical and pharmaceutical characteristics of microspheres obtained by the nanocomplex encapsulation method loaded with BMP-7. The composition of the microspheres comprised poly(lactic-co-glycolic acid) (PLGA), Tetronic (TR) and heparin (HP). The studied characteristics were: production yield, particle size and encapsulation efficiency (EE). Results are means ± SD, n=3.

Formulation	Theoretical loading (%)	Yield (%)	Size (μm) Mean ± SD	EE (%)
PLGA:TR/HP*	-	87.7 ± 6.2	26.0 ± 12.3	-
PLGA:TR/HP	0.01%	90.5 ± 3.6	21.6 ± 11.1	93.5 ± 4.8

* Control with a heparin amount corresponding to 0.01% loading.

FIGURE CAPTIONS

Figure 1. Scheme of the concept followed for the preparation of protein-loaded microspheres by the nanocomplex encapsulation method.

Figure 2. SEM images of microspheres obtained by the nanocomplex encapsulation method. (A) Blank microspheres corresponding to 0.2% w/w theoretical loading; (B) microspheres loaded with α-chymotrypsinogen (0.2% w/w theoretical loading); (C) blank microspheres corresponding to 0.01% w/w theoretical loading; (D) microspheres loaded with BMP-7 (0.01% w/w theoretical loading).

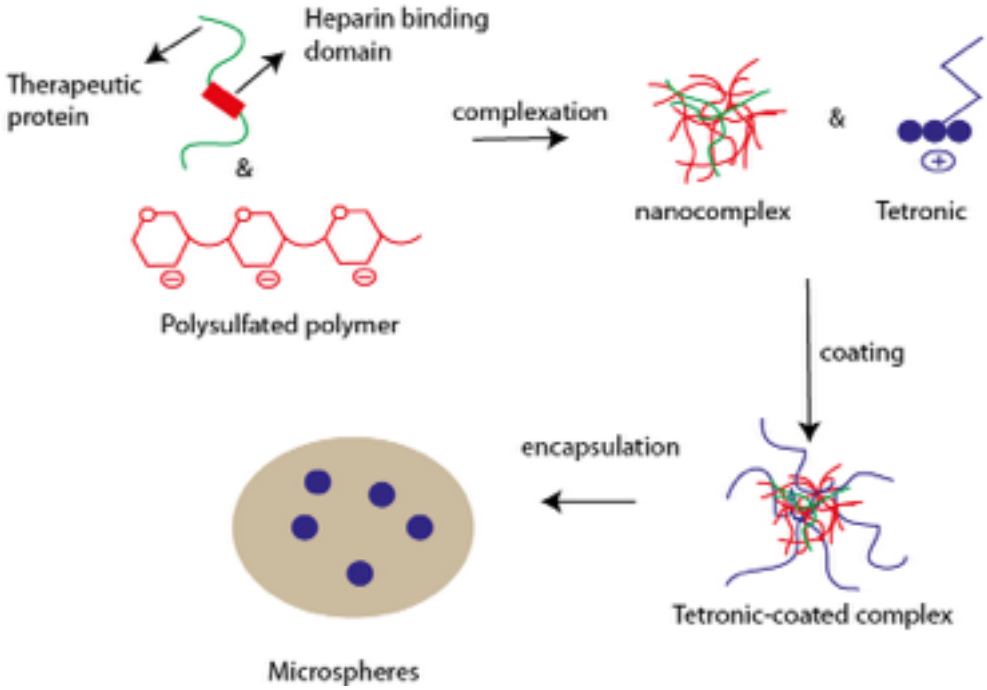
Figure 3. Release of BMP-7 from the microsphere formulation under *In vitro* conditions. The release experiments were performed in two different media: PBS containing 1% (w/v) BSA and mitogens-free neurosphere culture medium. Data represents means ±SD, n=3.

Figure 4. Effect of BMP-7 and the different components of the microspheres on the clonogenic index of U-87MG cells cultured as neurospheres (A) and on the size of these neurospheres (B). Effect of BMP-7 released from the microspheres at 30 and 90 days on the clonogenic index of U-87MG cells cultured as neurospheres (C) and on the size of these neurospheres (D). The size of the neurospheres was classified in small (area between 1450 and 5918 μm^2), medium (area between 5919 and 11340 μm^2) and large (area between 11341 and 85296 μm^2). The groups tested were: neurosphere culture medium (Control), heparin, Tetronic, soluble BMP-7 (BMP-7), a mixture of soluble BMP-7, heparin and Tetronic in culture medium (Mixture), BMP-7 released from the microspheres at 30 and 90 days (30dR, 90dR), release medium form blank microspheres at the same time points (30dC, 90dC), and release medium from blank microspheres mixed with soluble BMP-7 (90dC+BMP7). All samples were in neurosphere culture medium and BMP-7 concentration was always adjusted to 50 ng/mL. Data represents means ±SD, n=6.

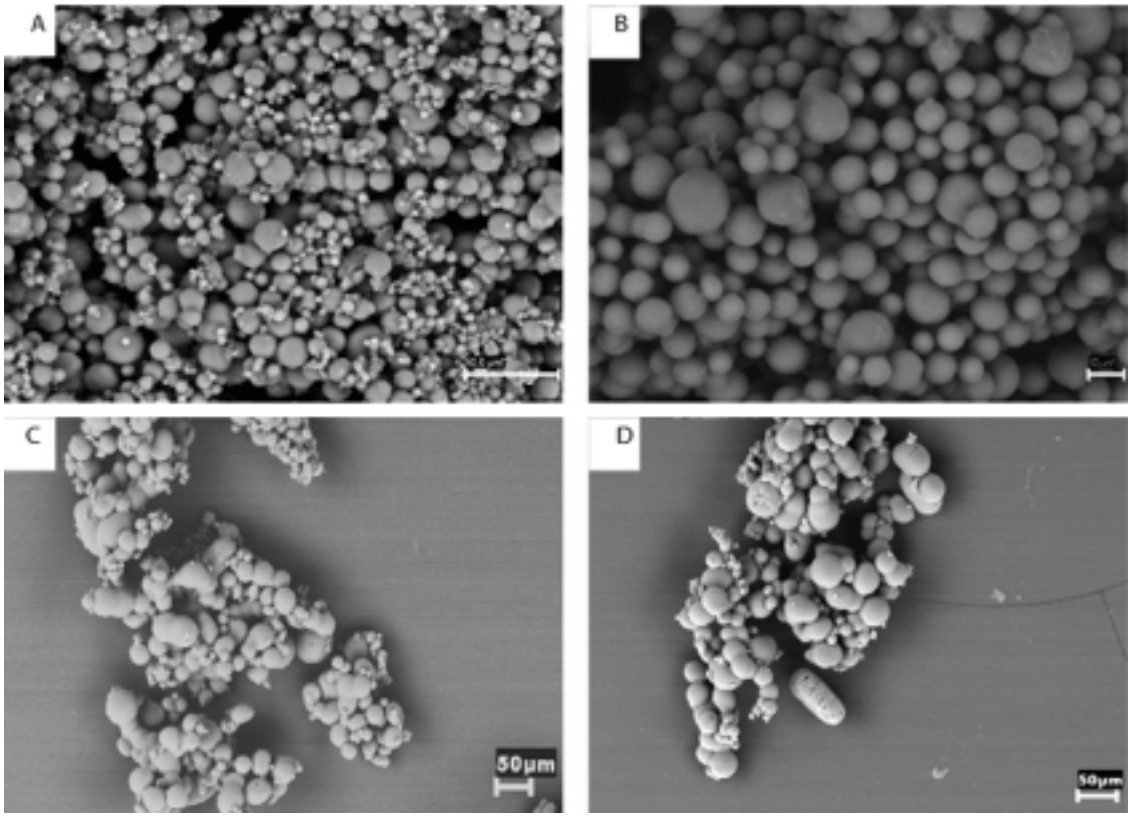
Figure 5. Optical microscopy images of U-87MG neurospheres upon (A) culture in neurosphere medium for 7 day (Control) or cultured for the same time in: (B) control medium with soluble BMP-7, (C) control medium preincubated at 37ºC in an incubator for 15 days, (D) release medium from blank microspheres (90 days, 90dC), (E) 90dC supplemented with soluble BMP-7, (F) release medium of BMP-7 loaded microsphere (90 days time point, 90dR). Arrows show cells that did not initiate neurosphere formation. All samples were in neurosphere culture medium, with BMP-7 concentration adjusted to 50 ng/mL.

Figure 6. Representative cell cycle/BrdU incorporation profiles of U-87MG neurospheres cultured in control medium (A) or in control medium with soluble BMP-7 (B).

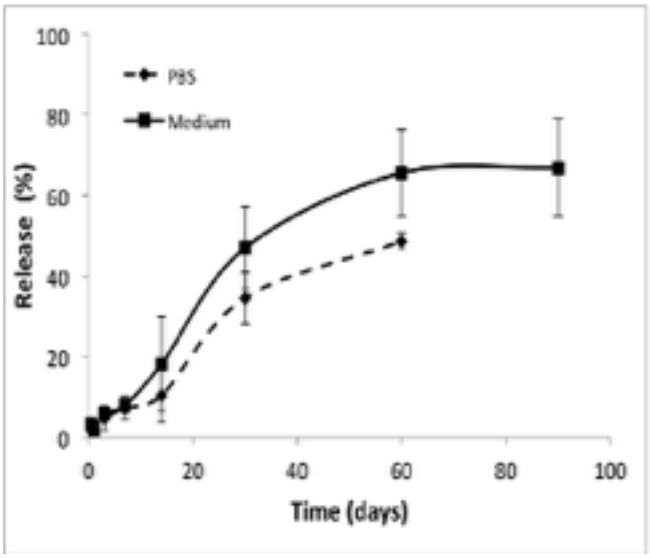
Reguera- Nuñez et al. **Figure 1**



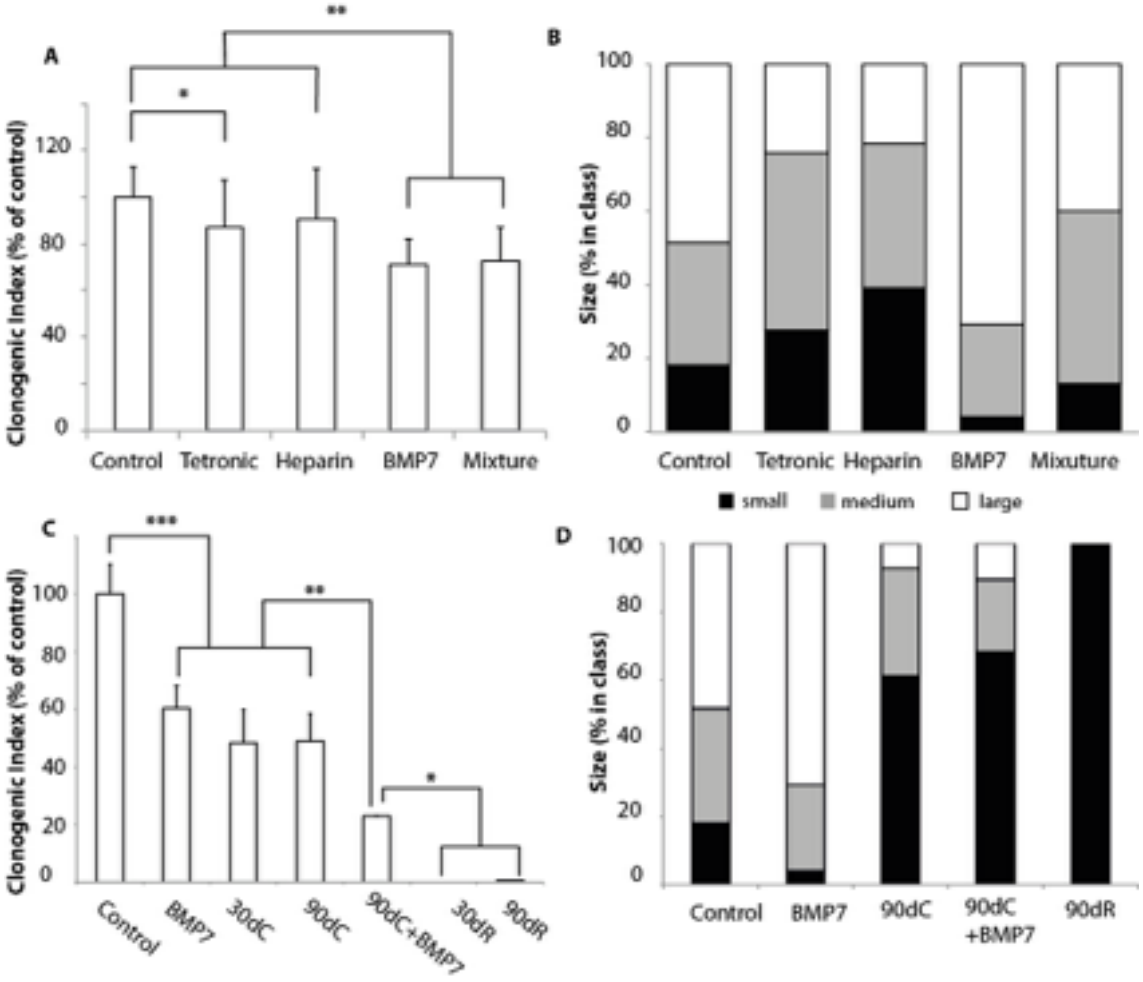
Reguera- Nuñez et al. **Figure 2**



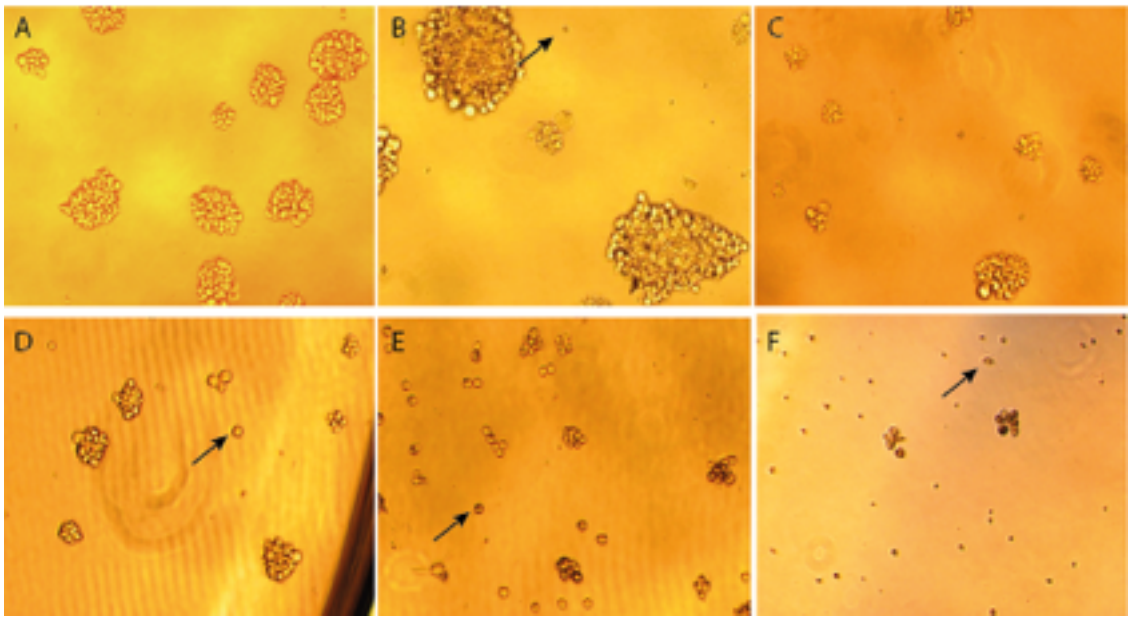
Reguera- Nuñez et al. **Figure 3**



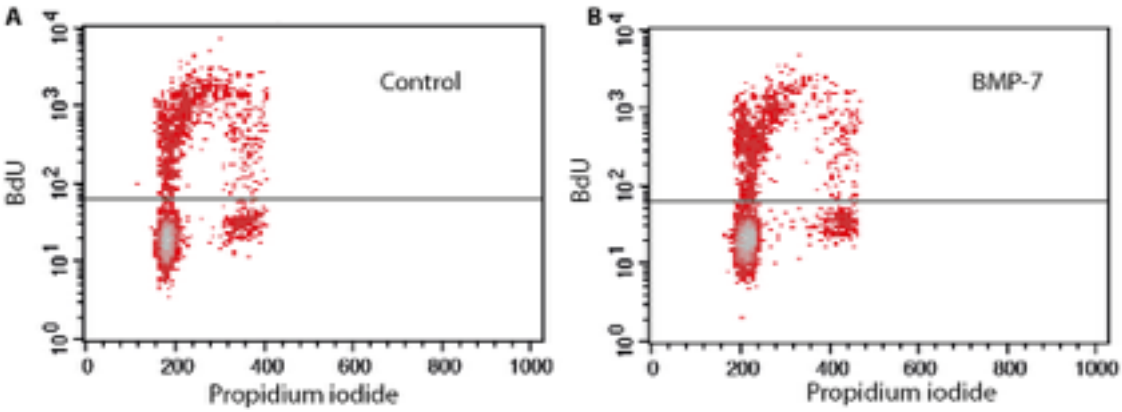
Reguera- Nuñez et al. **Figure 4**



Reguera- Nuñez et al. **Figure 5**



Reguera- Nuñez et al. **Figure 6**



1. SUPPLEMENTARY METHODS

1.1. Microsphere preparation by the double emulsion solvent-evaporation method

Microspheres were prepared by a water-in-oil-in-water (W/O/W) double emulsion solvent evaporation method. Briefly, 20 µg or 40 µg of α-chymotrypsinogen (for 0.1 and 0.2% w/w theoretical loading, respectively), and heparin (for a final α-chymotrypsinogen:heparin 1:1 mass ratio) were dissolved in 40 µL of ultrapure water. This aqueous solution was added to 400 µL of dichloromethane containing 20 mg of PLGA and 2 mg of Tetronic (Tetronic® T90R4, HBL=11, Mw=7.2 KDa, Sigma-Aldrich, Spain), and the resulting heterogeneous system was mixed by vortex (UniEquip, Model Zx3, Germany) at maximum speed to form a W/O emulsion. This primary emulsion was then added to 2 mL of 2% (w/v) polyvinyl alcohol (PVA) solution and mixed again by vortex to form a W/O/W double emulsion. This double emulsion was diluted with 8 mL of a 0.5% (w/v) PVA solution and magnetically stirred for 3 hours in an extraction hood. Afterwards, any remaining solvent was evaporated under reduced pressure (Buchi, Rotavapor R-210, Switzerland). The formulation was centrifuged at 7000 RCF for 10 minutes at 25ºC (Beckman Coulter, Microfuge 22R, Germany). Finally, the pellet was frozen and freeze-dried (primary drying: -35ºC, secondary drying: 25ºC; VirTis, Genesis 25ES, USA). The obtained microspheres were stored at 4ºC until used.

2. SUPPLEMENTARY DATA

Table 1. Physicochemical and pharmaceutical characteristics of microspheres obtained by the W/O/W double emulsion method loaded with α-chymotrypsinogen (0.1 and 0.2% w/w theoretical loadings). The composition of the microspheres comprised poly(lactic-co-glycolic acid) (PLGA), Tetronic (TR) and heparin (HP). The studied characteristics were: production yield, particle size and encapsulation efficiency (EE). Results are means ± SD, n=3.

Formulation	Theoretical loading (%)	Yield (%)	Size (μm)	EE (%)
PLGA	-	51.2±10.9	8.6±7.6	-
PLGA:TR	-	85.3±0.9	7.9±4.5	-
PLGA:TR:HP*	-	91.0±4.0	19.1±10.9	-
PLGA:TR:HP	0.1	89.6±3.7	10.3±6.3	39.8±0.6
PLGA:TR:HP**	-	92.0±2.7	15.8±8.8	-
PLGA:TR:HP	0.2	87.9±3.6	13.4±8.9	36.5±2.3

* Control with a heparin amount corresponding to the 0.1% loading formulation.
** Control with a heparin amount corresponding to the 0.2% loading formulation.

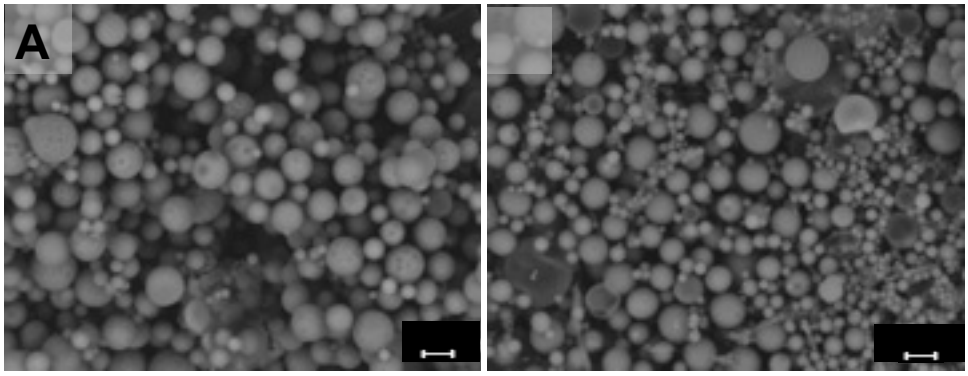


Figure S1. SEM images of microspheres obtained by the W/O/W double-emulsion evaporation technique. (A) Blank microspheres control for the 0.2% loading formulation. B) Microspheres loaded with α-chymotrypsinogen (0.2% w/w theoretical loading).

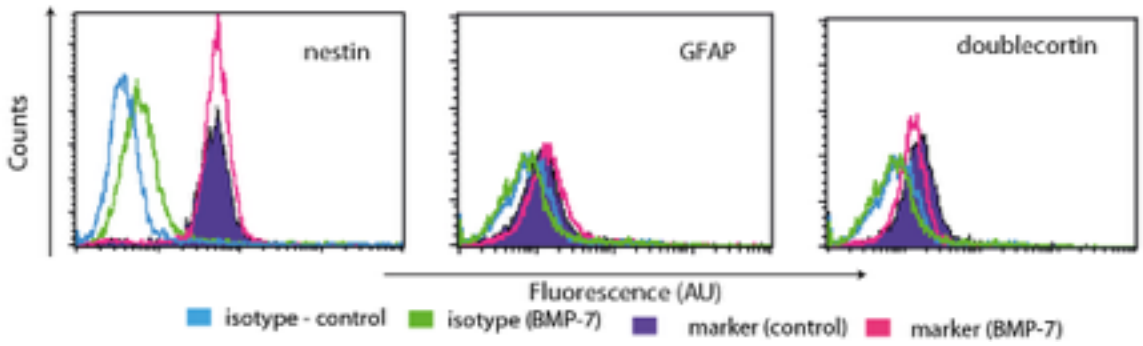


Figure S2: Surface markers of U-87MG cells. Cells were cultured as neurospheres in control medium or under the same conditions but supplemented with soluble BMP-7. For comparison we include negative isotype controls treated and not treated with BMP-7. The concentration of BMP-7 was always adjusted to 50 ng/mL. Data shows representative results from FACS analysis.