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Enhanced Bioactivity of α -Tocopheryl Succinate Based Block Copolymer Nanoparticles by Reduced Hydrophobicity

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Supporting Information

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Abstract

Well-structured amphiphilic copolymers are necessary to obtain self-assembled nanoparticles (NPs) based on synthetic polymers. Highly homogeneous and monodispersed macromolecules obtained by controlled polymerization have successfully been used for this purpose. However, disaggregation of the organized macromolecules is desired when a bioactive element, such as α -tocopheryl succinate, is introduced in self-assembled NPs and this element must be exposed or released to exert its action. The aim of this work is to demonstrate that the bioactivity of synthetic NPs based on defined reversible addition-fragmentation chain transfer polymerization copolymers can be enhanced by the introduction of hydrophilic comonomers in the hydrophobic segment. The amphiphilic terpolymers are based on poly(ethylene glycol) (PEG) as hydrophilic block, and a hydrophobic block based on a methacrylic derivative of α -tocopheryl succinate (MTOS) and small amounts of 2-hydroxyethyl methacrylate (HEMA) (PEG-*b*-poly(MTOS-*co*-HEMA)). The introduction of HEMA reduces hydrophobicity and introduces “disorder” both in the homogeneous blocks and the compact core of the corresponding NPs. These NPs are able to encapsulate additional α -tocopheryl succinate (α -TOS) with high Efficiency and their biological activity is much higher than that described for the unmodified copolymers, proposedly due to more efficient degradation and release of α -TOS, demonstrating the importance of the hydrophilic–hydrophobic balance.

Abstract

Keywords

amphiphilic polymers; anticancer nanoparticles; HEMA; RAFT polymerization; terpolymers; α -tocopheryl succinate

1. Introduction

Currently available cancer therapies based on chemotherapeutic drugs suffer from their inability to differentiate between cancerous versus healthy cells as well as their inappropriate

pharmacokinetics and nonspecific biodistribution.^[1,2] Significant deleterious side effects and drug resistances are also becoming more frequent.^[3] Nanotechnology, and more specifically surfactant free polymeric nanoparticles (NPs), have emerged as a potent new class of anticancer drug delivery vehicles with higher activities, selectivities, and lower toxicities than many existing drugs.^[4,5] Such nanovehicle-based platforms have multiple advantages that can overcome the current limitations of conventional anticancer treatments, for example, by smartly responding to the heterogeneous and complex environment inside the tumors.^[3,6,7]

NPs based on well-structured amphiphilic polymers have been obtained by self-assembly process via phase-inversion. This process is governed by a delicate balance between hydrophobic and hydrophilic interactions and it is especially relevant when the amphiphilic polymer includes bioactive elements (e.g., drugs) covalently attached to its structure (polymer drug) to avoid premature leaching and release of the drug.^[8,9] If the drug is hydrophobic and forms part of a hydrophobic block, it will be concentrated in the core of the nanoparticles when suspended in water, and the disaggregation of the organized macromolecules will be necessary to expose or release the active principle and exert its action.^[10] The hydrophobic interactions between hydrophobic blocks lead to the chain organization in polymeric particles, and the hydrophilic repulsion contribute to the stabilization of the particles and to facilitate water diffusion to the interior of the NPs, that may play a critical role in chain disaggregation resulting hydrolysis of cleavable linkers and drug delivery.^[11,12]

There are numerous reports in the literature describing the synthesis and characterization of amphiphilic polymeric nanocarriers for the treatment of cancer.^[12–14] However, there are not so many examples describing how the structure modification of one segment affects the bioactivity of the particle. In this sense, Zhang et al. prepared NPs of poly(styrene-*b*-ethylene-*co*-butylene-*b*-styrene) by microphase inversion and studied the effect of different concentrations of carboxylic groups in the styrene residues. They observed that the NPs gradually changed from a uniform and compact globule to a hyperbranched and loose cluster as the extension of the carboxylation increased. Moreover, the cluster was broken by simple dilution of the suspension.^[15] Additionally, Akiyoshi et al. described how amphiphilic cholesteryl group-bearing pullulans formed nanogels in dilute conditions due to the hydrophobic interactions between cholesteryl groups.^[16] Later, these authors introduced cationic groups in the cholesteryl group-bearing pullulans (cCHP) to efficiently deliver proteins to myeloma cells and primary CD4⁺ T lymphocytes by induction of macropinocytosis. The complex of the nanogel-protein dissociated and the protein was released inside the cell.^[17]

Our group described the “progressive accessibility model” to explain the zero-order release of hydrophobic drugs from copolymer drugs based on a hydrophobic monomer (a methacrylic derivative of Triflusal, THEMA) and an ionic hydrophilic comonomer (2-acrylamido-2-methylpropano sulfonic acid, AMPS) randomly distributed and obtained by free radical copolymerization (poly(THEMA-*co*-AMPS)).^[18] The article emphasized the importance of the presence of the hydrophilic monomer (AMPS) in the Triflusal release

process. AMPS warranted the water accessibility to the ThEMA residues and favored its hydrolysis and the release of the antithrombogenic compound.

Moreover, our group also described the formation of oriented micelles by an amphiphilic copolymeric system based on butylacrylate (BA) and 5-methacrylamide-2-naphthalenesulfonic acid (MANSA) (poly(BA-*co*-MANSA)), and established that its antiangiogenic activity was related with the self-assembled micellar morphology of the nanoparticles, which placed the sulfonic groups in an adequate position to interact and inhibit fibroblast growth factor.^[19]

The aim of this paper is to demonstrate that small changes in the delicate hydrophilic–hydrophobic balance of amphiphilic polymer drugs (by the introduction of small amounts of a hydrophilic comonomer in the hydro-phobic block) are important not only in the self-assembly process of the NPs, but also in the control of the bioactivity of the polymer drug and the correspondent NP. For this purpose, we obtained reversible addition-fragmentation chain transfer polymerization (RAFT) block copolymers based on poly(ethylene glycol) (PEG) as hydrophilic block, and a hydrophobic block based on a methacrylic derivative of α -tocopheryl succinate (MTOS) and small amounts of 2-hydroxyethyl methacrylate (HEMA) randomly copolymerized (Figure 1).

α -Tocopheryl succinate (α -TOS) is a mitochondrially targeted anticancer compound (mitocan)^[20] that selectively induces apoptosis of cancer cells and regulates angiogenesis by inducing apoptosis of proliferating endothelial cells with no harm to quiescent endothelial cells.^[21–23] However, the hydrophobicity of this drug significantly limits its real application in cancer treatments and therefore, the design of optimal and efficient drug delivery systems of α -TOS are an important goal to achieve.^[24,25]

Our group has recently described new bioactive self-assembled NPs based on amphiphilic copolymers bearing α -TOS with antiproliferative and antiangiogenic properties, and able to encapsulate hydrophobic molecules in the core. These copolymers were obtained by free radical polymerization of monomers with different hydrophilicity and reactivity^[26] or by RAFT.^[27] In this sense, RAFT is considered one of the most versatile living/controlled radical polymerization as it allows the design and synthesis of functional polymers with a well-defined architecture and controlled molecular weight (MW) with relatively low polydispersity ($\bar{D}$).^[28,29] The structural homogeneity and the amphiphilic nature of the obtained macromolecules allows the self-assembly in aqueous media to form 3D supramolecular structures when the adequate hydrophilic/hydrophobic balance is reached.

Although the RAFT-based block copolymers (PEG-*b*-PMTOS) presented higher content of α -TOS than the free radical polymerized copolymers (poly(*N*-vinyl pyrrolidone-*co*-MTOS)),^[26] their biological activity was significantly lower, probably due to the so called “PEG dilemma”^[30,31] and the high stability of the NPs obtained by these highly homogeneous block copolymers resulting from too strong hydrophobic interactions making the core impermeable for water. Therefore, the new synthetic terpolymers described in this paper were designed in order to reduce the hydrophobicity of the hydrophobic block by introducing the hydrophilic comonomer HEMA (Figure 1) to favor the water diffusion in the

core of the NP, the disaggregation of the macromolecules forming the NP, and the release of the loaded cargo.

2. Results and Discussion

2.1. RAFT Polymerization: Characterization of PEG-*b*-[Poly(MTOS-co-HEMA)]

PEG-*b*-polyMTOS block copolymers with a molar composition of PEG:MTOS 66:34, 71:29, 76:24, 82:18, and 87:13 were previously reported and are labeled as PEG-66, PEG-71, PEG-76, PEG-82, and PEG-87.^[27]

In order to change the structural homogeneity of these block copolymers and analyze the resulting effect on the biological activity, HEMA was copolymerized with MTOS (10 and 20 mol% with respect to MTOS) using the macro-chain transfer agent PEG-CTA₈ (MW of 8 kDa) at 0.5 M and 70 °C during 5 h. Other experimental conditions are listed in Table 1. The macro chain transfer agent (CTA), PEG-CTA₈, was selected due to its high compatibility toward meth-acrylic monomers and its optimal molar mass, avoiding their uptake by the reticuloendothelial (RES) system while still allowing clearance by renal filtration (MW cut-off for glomerular filtration is around 30 kDa).^[32–34] HEMA was added to the terpolymers due to its excellent biocompatibility and hydrophilicity of the resulting polymer polyHEMA. Additionally, polyHEMA has been approved by the FDA for biomedical and pharmaceutical applications.^[35–38] The methacrylic group of HEMA is linked to a hydroxyl ethyl moiety that is also present in the MTOS monomer. Therefore, the similar chemical structure in the proximity of the double-bond is expected to provide similar reactivity to both monomers, and a statistical copolymer will be formed with close to random distribution of HEMA in the MTOS hydrophobic block. Moreover, the HEMA monomer was also chosen because it is the chemical residue that will result from the hydrolysis of MTOS by esterases from body fluids and the release of α-TOS. The complete hydrolysis of PEG-*b*-polyMTOS will thus give rise to the hydrophilic block copolymer PEG-*b*-polyHEMA.^[39] The terpolymers were labeled PEG-YH where Y indicates the feed molar composition of HEMA and their characteristics are shown in Table 1.

The HEMA copolymerization was confirmed by ¹H-NMR by the disappearance of the signals of vinyl protons corresponding to its methacrylate group at 5.58 ppm and comparison with that of MTOS protons at 5.55 ppm. The MTOS units into the macromolecular chains were elucidated by ¹H-NMR^[27] and the HEMA units by considering that 100% of the conversion was reached as the signal at 5.58 ppm disappears during the polymerization reaction. As a result, terpolymers with molar composition PEG:MA (MA is MTOS+HEMA) of 76:24 and 80:20 were obtained. Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectra of PEG-10H and PEG- 20H showed a characteristic O–H stretching vibration band of HEMA at 3500 cm^{−1} (Figure 2), also confirming the successful incorporation of HEMA units into the macromolecular chains.

Size exclusion chromatography (SEC) characterization revealed that both terpolymers had a relatively narrow MW distribution with Đ < 1.26. The terpolymerization kinetics are summarized in Figure 3, showing the evolution of Ln([M]₀/[M]_t) and M_n values as a function of reaction time and MTOS conversion, respectively. In both cases, linear first order

kinetic plots were obtained that is an indicative a constant free radical concentration and, thus, demonstrates the absence of significant termination reactions while the linear increase of M_n with conversion confirms fast initiation and absence of significant irreversible transfer reactions. Additionally, normalized SEC traces at different polymerization times are compared in Figure 3C,D. As expected, the traces were clearly shifted to lower elution times, indicating an increase in the molecular weight, with increasing reaction time.^[40] It is noteworthy that traces were bimodal, due to the presence of a minor fraction of unreacted macro at 24 min. This behavior is commonly observed for this type of polymerizations, indicating that the efficiency of macro CTA agents was not complete.^[41,42] Non-normalized SEC traces (Figure S1, see Supporting Information) clearly show that the CTA peak is small and constant over time. The purification of copolymers by dialysis effectively eliminated the majority of low MW chains of the unreacted PEG macro-CTA agents (Figure S2, see Supporting Information).

These results confirmed that the copolymerization with HEMA allowed to maintain the control of the polymerization. The HEMA monomeric units were presumably incorporated randomly into the MTOS based hydrophobic block due to a similar reactivity of both methacrylic monomers, as previously commented. These structural modifications affected the self-assembly behavior of the copolymers and their therapeutic action, as will be demonstrated in the next sections.

2.2. Characterization of Self-Assembled NPs

The incorporation of low concentrations of HEMA into the hydrophobic block of PEG-*b*-poly(MTOS-*co*-HEMA) allowed to obtain spherical NPs by self-organized precipitation (SORP) or solvent exchange as indicated by the very low polydispersity index (PDI) values resulting from dynamic light scattering (DLS).^[43,44] These NPs were labeled as NP-YH where Y indicates the feed molar composition of HEMA and their characteristics are shown in Table 2.

Figure 4A shows the size distributions of NP-10H and NP-20H. Both distributions were narrow and unimodal, as revealed by PDI values lower than 0.06. D_h of 104 and 144 nm were obtained from NP-10H and NP-20H, respectively. NP-10H presented a smaller size in comparison to NP-20H. This effect could be due to a balance between the formation of hydrogen bonds through hydroxyl groups of HEMA and the alteration of the well-defined structure of the block copolymers. This behavior was also observed by Zhang et al. for carboxylated poly(ST-*b*-ethylene-*co*-butylene-*b*-ST). D_h decreased with low concentrations of carboxylic groups and then increased when the concentration of carboxylic groups raised.^[15] These sizes are quite appropriate for their application as drug delivery system for cancer treatment, avoiding the RES system and improving the uptake by cells. Slightly negative zeta potentials were obtained for all NPs, indicating a nearly neutral charge of the surface. This behavior has also been observed in other PEG nanoassemblies.^[45] Additionally, scanning electron microscopy (SEM) micrographs (Figure 4B) confirmed the spherical morphology of these nanoassemblies.

2.2.1. Encapsulation Efficiency (EE): α -TOS-Loaded NP—The described block copolymers are excellent candidates to encapsulate hydrophobic molecules in their inner core. α -TOS-loaded NPs (10% w/w respect to the polymer) were successfully prepared and characterized and the results are summarized in Table 2. Particularly, EE was higher than 75% in all copolymeric systems. The EE was especially improved for the NPs with a high PEG content (EE higher than 94%), probably as a result of the protection and stabilization of the hydrophobic core by the PEG shell.^[33]

All size distributions were unimodal with low PDI values. Furthermore, the hydrodynamic diameters were lower than 155 nm. NP-10H+ α -TOS were significantly smaller than those based on NP-20H+ α -TOS (Figure 4C). α -TOS-loaded NPs presented slightly higher D_h and PDI values in comparison to analogous unloaded NPs. Moreover, no appreciable differences in the zeta potentials of unloaded versus α -TOS-loaded NPs were found. Additionally, the size of α -TOS-loaded NPs based on block copolymers PEG-*b*-polyMTOS decreased as a function of PEG content into macromolecular chains. As an example, size distributions of loaded NPs based on PEG-71 and PEG-87 are compared in Figure 4E. It is noteworthy that the distribution curves were narrow, unimodal, and were clearly shifted to lower D_h as a result of the stabilization of PEG chain into the shell of the nanoassemblies. SEM micrograph of loaded NPs from NP-87+ α -TOS (Figure 4F) revealed the well-defined spherical morphology of the NPs. No aggregation was observed at room temperature.

2.3. Uptake and Colocalization of Coumarin-6-Loaded NP

Coumarin-6 (c6) as a fluorescent probe was efficiently entrapped in the core of the NPs (EE higher than 89%), obtaining NP sizes between 115 and 160 nm and a nearly neutral charge on their surface (Table 2). These fluorescent NPs were used to trace their cellular uptake by MDA-MB-453 cells. Figure 5A shows the evolution of green fluorescence intensity in cancer cells, including confocal micrographs after 0.5, 2, and 6 h of incubation with NP-10H+c6. These results demonstrated that c6-loaded NPs were able to cross the cellular membrane of cancer cells in only 30 min. However, the intensity of fluorescence increased progressively after 2 and 6 h of NP treatment. Additionally, confocal images proven that the NPs were located inside the cytoplasm of cells, around the nucleus. According to the particle size of c6-loaded NPs, the endocytosis could be the most probable mechanism of NP uptake within cancer cells.^[46]

After 6 h, mitochondria of cancer cells were stained with MitoTracker Red CMXRos in order to examine the ability of c6-loaded NPs to accumulate inside these organelles. Confocal micrographs of the colocalization study using NP-10H+c6 and NP-20H+c6 are shown in Figure 5B,C, respectively. Bright orange fluorescence was observed as a result of the composition of green and red fluorescent images, confirming the colocalization of c6-loaded NP into the mitochondria of cancer cells. According to the morphology of the nanoparticles, the surface is essentially hydrophilic and constituted by PEG chains. c6 is incorporated in the hydrophobic core formed by copolymer segments of MTOS and low amounts of HEMA. It is possible that the diffusion of the fluorescent c6 should be partially produced in the interval of the endocytosis, but from an experimental point of view, the MitoTracker assay represented in Figure 5B,C demonstrates that there is an evident

accumulation of the core-shell nanoparticles in the mitochondria. Further experiments are being carried out with specific targeting components to investigate the interaction of the functionalized nanoparticles with the mitochondria.

α -TOS acts as a competitive inhibitor of succinate dehydrogenase (complex II) by displacing ubiquinone (coenzyme Q) from binding to complex II in the cancer cell mitochondria.^[47,48] Therefore, the localization of the synthesized NPs inside this organelle could enhance their biological activity.

2.4. Biological Activity

The therapeutic activity of all NPs was tested by Alamar Blue assay using two different breast cancer cells: luminal A human metastatic carcinoma (MCF-7) and HER2 positive human breast adenocarcinoma (MDA-MB-453) cells.^[49] It should be noted that physical mixtures of block copolymer nanoparticles with free α -TOS could not be utilized as control due to insolubility of α -TOS in PBS buffer. Moreover, the NPs loaded with α -TOS were prepared with 10% (w/w) with respect to polymer and the drug loading of the HEMA containing copolymers will also be slightly lower compared to the pure PEG-polyMTOS block copolymers. However, these changes are less than 10% in drug loading and, therefore, we have chosen to discuss the biological activity based on NP concentration rather than amount of α -TOS in the following.

α -TOS-loaded NPs based on PEG-*b*-PMTOS were significantly more active than unloaded NPs (Figure 6). The biological activity of loaded NPs significantly increased as a function of the NPs concentration and MTOS content of the block copolymers. All NP formulations reduced the viability below 70% at the highest NPs concentration. In fact, cell viability was lower than 40% for NP-71 and NP-76, and 60% for NP-82 and NP-87. Additionally the significant cytotoxicity of the different NPs formulations was observed at lower concentrations until 0.63 mg mL⁻¹ in comparison to unloaded NPs. The improvement of the antiproliferative activity of MTOS-based NPs was more significant in the case of NP-82 and NP-87 with a high PEG content.

The most active loaded NPs (NP-71+ α -TOS) was selected to analyze its effect on MCF-7 viability over 72 h. In fact, the presence of additional α -TOS enhanced the cytotoxicity of NP-71 formulation and this effect was more pronounced after 48 and 72 h (Figure 7). In particular, the viability of unloaded NPs was not reduced below 70% within the timeframe of experimentation and a slight decrease in cell viability was only observed between 48 and 72 h. In contrast, the effect of α -TOS-loaded NPs was significantly higher after 48 h, as the viability was reduced from 75% at 24 h to 35% at 48 h, probably due to the release of the drug into the cancer cells during this period of time. It is noteworthy that MCF-7 cells were less sensitive than MDA-MB-453 to the presence of NPs. This difference could be probably attributed to the metabolic characteristics of MDA-MB-453 and MCF-7, regarding to the alteration of their mitochondria and the relative importance of glycolic metabolism for each cell line.^[50,51]

The effect of the incorporation of HEMA into the hydrophobic polyMTOS block on the anticancer activity of polymeric formulations was evaluated on MCF-7. Figure 8A shows

that the therapeutic action of these NPs greatly increased with the incorporation of HEMA monomeric units. The viability of MCF-7 cells decreased to 49% and 68% after 24 h for NP-10H and NP-20H, respectively. Furthermore, this effect was more evident at longer periods of time, reaching viabilities lower than 40% after 72 h.

The enhancement of therapeutic action of polymeric systems as a result of HEMA incorporation can be explained by considering the modification of hydrophobicity of MTOS based-core of NPs. In absence of HEMA, the precise control of the macromolecular architecture gave rise to well-organized stable nanoassemblies. However, the increase of the hydrophilicity of hydrophobic block, favored the therapeutic action of NPs,^[18,52] probably due to the enhancement of the water diffusion and the hydrolysis rate that represent crucial factors for the release of α -TOS from NPs by hydrolysis as we recently demonstrated for related acid-degradable thermoresponsive polymers.^[53]

Unexpectedly, NP-20H (with higher content of HEMA) had a lower effect on cell viability than NP-10H, possibly due to a decrease in MTOS units into the hydrophobic block copolymers. For that reason, it maybe speculated that NP-10 had a better balance between the number of active MTOS and HEMA units that were randomly distributed in the hydrophobic block.

The most active NPs based on PEG-10H were also evaluated against MDA-MB-453 and the effect of loading of α -TOS in the core of the NPs was also studied. Figure 8B shows the MDA-MB-453 cell viability after the treatment of different concentrations of unloaded and loaded NP-10H. The loading of additional α -TOS into the core of NPs based on PEG-10H significantly improved their anticancer activity. In fact, cell viability was reduced to 20% at 2.5 and 1.25 mg mL⁻¹ after 24 h. Additionally, the NPs were found to be cytotoxic down to concentrations of 0.31 mg mL⁻¹.

α -TOS has demonstrated selectivity against cancer cells. However, the mechanisms involved in this selectivity are not well understood at the present. The most commonly accepted theory to explain this behavior is based on the difference of cellular metabolism between cancer and healthy cells, which is related to the levels of esterase and antioxidants enzymes and the mechanism to produce ROS.^[54,55] In particular, the levels of these enzymes are diminished in cancer cells, allowing the accumulation of ROS species that stimulate the release of cytochrome C and therefore trigger apoptosis.^[56,57] Furthermore, the negative charge of α -TOS at neutral pH could also be correlated with its selective anticancer activity due to the acidic pH of most solid tumors (around 6.0–6.5), while pH is neutral in the case of the healthy tissues.^[58] For that reasons, the biological activity of NP-71 and NP-71+ α -TOS was also measured against nontumoral HMEpiC cells in order to know if these nanoassemblies preserve α -TOS selectivity toward breast cancer cells (Figure 9).

Nontumoral HMEpiC cells were significantly less sensitive to the presence of the NPs. In fact, viability of HMEpiC cells was higher than 70% in the presence of unloaded NPs (figure 8A,B). In the case of loaded NPs, the viability of HMEpiC only decreased below 70% at high NP concentrations. However, NP-10H+ α -TOS reduced MDA-MB-453 cancer cell viability to 20% at 2.5 mg mL⁻¹ after 24 h, while the HMEpiC cell viability was maintained

very close to 70%. This formulation presented a high activity and a selective interval from 0.31 till 2.50 mg mL⁻¹.

3. Experimental Section

3.1. Materials

HEMA (Sigma-Aldrich) was purified according to the literature.^[59] 2,2'-Azobisisobutyronitrile (AIBN, Merck) was recrystallized from methanol (m.p. 104 °C). Anhydrous 1,4-dioxane (Sigma-Aldrich), deuterated chloroform (CDCl₃, Sigma-Aldrich) and chromatographic grade tetrahydrofuran (THF, Sigma-Aldrich) were used without further purification to synthesis and characterize polymeric systems. Additionally, sodium chloride (NaCl, Panreac), α -tocopherol succinate (α -TOS, Sigma-Aldrich) and coumarin-6 (c6, Sigma-Aldrich) were used without further purification to prepare self-assembled NPs.

3.2. Characterization Techniques

¹H-NMR was performed in a Mercury 400BB apparatus, operating at 400 MHz and dissolving the corresponding sample in CDCl₃ at 25 °C. FTIR-ATR spectroscopy was obtained in a Perkin Elmer Spectrum One FTIR spectrometer using 32 scans, and a resolution of 4 cm⁻¹. Thermogravimetric analysis were performed using a TGA Q500 apparatus (TA instruments), under dynamic nitrogen atmosphere between 25 and 600 °C at a heating rate of 10 °C min⁻¹ in order to analyze the thermal degradation of the polymeric systems. The number and weight average molecular weight (M_n and M_w) and $\bar{D}$ of the polymers were determined by SEC, using a Perkin-Elmer Isocratic LC pump 250 coupled to a refraction index detector (Series 200). Three polystyrene-divinyl benzene columns (PLgel, Varian, Polymer Laboratories) of average pore size of 10³, 10⁴, and 10⁵ Å were used as solid phase, and degassed THF (1 mL min⁻¹) was used as eluent at 40 °C. Mono-disperse poly(methyl methacrylate) (PMMA) standards (Scharlab) with molecular weights between 10 300 and 1 400 000 Da were used to obtain the calibration curve. Data were analyzed using the Perkin-Elmer LC solution program.

3.3. Preparation of Terpolymers PEG-*b*-[poly(MTOS-co-HEMA)]

The methacrylic derivative of α -TOS (MTOS), the PEG macro-CTA agents (4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid was conjugated to PEG with M_n between 8 and 22 kDa) and PEG-*b*-polyMTOS were obtained as previously described.^[26,27]

PEG-*b*-[poly(MTOS-co-HEMA)] were obtained by RAFT polymerization using PEG-macro CTA of 8 kDa MW (CTA-PEG₈). Two different feed molar ratios of methacrylic monomers were used, particularly 10 or 20 mol% HEMA with respect to MTOS. In this way, CTA-PEG₈, MTOS, HEMA, AIBN, and anhydrous 1,4-dioxane were sealed in a 25 mL Schlenk tube. The reaction mixture was degassed by three freeze-pump-thaw cycles and heated in an oil bath under magnetic stirring at 70 °C. The total monomer concentration ([M]), the reaction time and the feed molar ratio [MTOS]:[CTA]:[AIBN] were 0.5 M, 300 min, and [100]:[1]:[0.3], respectively

Samples (250 μL) were taken from the polymerization after different reaction times (30, 60, 120, and 300 min) in order to analyze the polymerization kinetics. These samples were cooled and characterized by SEC to determine the molecular weight and $^1\text{H-NMR}$ spectroscopy to calculate the composition of copolymer chains and conversion of the reaction. At the end of the polymerization reactions, terpolymers were purified by dialysis (Spectrum Laboratories, 25KDa MW cut-off) against a mixture of THF and water (50:50 v:v) that was progressively replaced by water during 3 days with the aim to remove the residual unreacted monomers and low molecular weight species. The resulting solutions were isolated by freeze-drying to yield white amorphous powders.

3.4. Self-Assembled NPs by SORP

Unloaded NPs were prepared by SORP or solvent exchange as described before.^[27,43] Briefly, an aqueous solution of NaCl ($100 \times 10^{-3} \text{ M}$, 2 mL) was added drop-wise to the organic solution of the polymer (10 mg mL^{-1} in dioxane, 2 mL) under constant magnetic stirring. The final polymer concentration was 5 mg mL^{-1} . Furthermore, the preparation of loaded NPs was optimized by the addition of the appropriate amount of α -TOS (10% w/w respect to the polymer) or c6 (1% w/w respect to the polymer) to the organic phase. Milky NPs dispersions were dialyzed against NaCl during 72 h in order to remove organic solvent and unloaded α -TOS or c6. Each NP suspension was then sterilized by filtration through $0.22 \times 10^{-6} \text{ M}$ polyethersulfone membranes (PES, Millipore Express, Millex GP) and stored at 4°C .

3.4.1. EE—NPs were freeze dried (yield higher than 80%) in order to calculate the EE of α -TOS or c6, that can be defined as the ratio of calculated and theoretical amount of the drug entrapped in the NPs. The calculation equation is as follows

$$\text{Encapsulation efficiency (EE)} = \frac{[\text{loaded molecule}]_i}{[\text{loaded molecules}]_o} \times 100 \quad (1)$$

Being $[\text{loaded molecule}]_i$ the concentration of the α -TOS or c6 encapsulated in the inner core of the NPs and detected experimentally, and $[\text{loaded molecule}]_o$ the concentration of the molecule added in the nanoprecipitation process.

In both cases, loaded NPs were dissolved in ethanol. The EE of α -TOS was quantified by absorbance spectroscopy ($\lambda = 285 \text{ nm}$) using a Perkin Elmer Lambda 35 UV/VIS spectrophotometer (calibration curve using α -TOS concentrations between 1 and 0.001 mg mL^{-1} in ethanol). Additionally, c6 entrapped in the NPs was quantified by fluorescence spectroscopy ($\lambda_{\text{excitation}} = 485 \text{ nm}$ and $\lambda_{\text{emission}} = 528 \text{ nm}$) using a Biotek SYNERGY-HT plate reader, adjusting emission splits as required for the intensity measurements (calibration curve was obtained using c6 concentrations between 0.5 and 0.001 mg mL^{-1} in ethanol).

3.4.2. NP Characterization—The morphology of NPs was investigated by SEM using a Hitachi SU8000 TED, cold-emission FE-SEM microscope working with an accelerating voltage between 25 and 50 kV. Samples were prepared by deposition of one drop of the NP suspension (0.05 mg mL^{-1}) over small glass disks (12 mm diameter). After the evaporation

of the aqueous phase overnight, the samples were coated with a gold palladium alloy (80:20) prior to examination by SEM.

The hydrodynamic diameter (D_h , by intensity) and PDI of unloaded and α -TOS entrapped NPs was determined by DLS using a Malvern Nanosizer NanoZS Instrument equipped with a 4 mW He-Ne laser ($\lambda = 633$ nm) at a scattering angle of 173° . Different samples were measured in square polystyrene cuvettes (SARSTEDT) at 25°C . The autocorrelation function was converted in an intensity particle size distribution with ZetaSizer Software 7.10 version, based on the Stokes-Einstein equation. The zeta potential was quantified by laser Doppler electrophoresis (LDE) using NPs formulation at 0.5 mg mL^{-1} in $10 \times 10^{-3}\text{ M NaCl}$. The zeta potentials were automatically calculated from the electrophoretic mobility using the Smoluchowski's approximation. For each sample, the statistical average and standard deviation of data were calculated from 8 measurements of 20 runs each one.

3.5. Biological Activity

3.5.1. Cell Culture—Human mammary adenocarcinoma cells, MCF-7 cells (European Collection of Authenticated Cell Cultures (ECACC)), were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, 1% penicillin/streptomycin (PS) and incubated at 37°C and 5% CO_2 . Additionally, human metastatic carcinoma cells, MDA-MB-453 cells (American Type Culture Collection (ATCC)), were cultured in DMEM, supplemented with 10% FBS, 1% PS and incubated at 37°C and 5% CO_2 . Finally, human mammary epithelial cells, HMEpiC cells (obtained from Innoprot, P10891), were cultured using mammary epithelial cell medium (MepiCM, Innoprot), at 37°C and 5% CO_2 .

3.5.2. Uptake and Intracellular Location of c6 Loaded NPs—Endocytosis of c6 loaded NPs was followed by confocal laser scanning microscopy (CLSM) (Leica TCS-SP5 RS AOBS). For this propose, MDA-MB-453 cells were seeded into 24 well plates at $100\,000\text{ cells mL}^{-1}$, in complete medium. The cells were incubated for 48 h at 37°C . Afterward, the medium was replaced with the corresponding NPs dispersion in NaCl (500 μL of the NPs suspension and 500 μL of completed medium) and incubated at 37°C . At different time points (30 min, 2 and 6 h), the cells were washed three times with cold PBS and fixed by a paraformaldehyde solution in PBS (3.7% w/v) for 15 min at room temperature. Then, the cells were rinsed with cold PBS and observed by CLSM. The fluorescence intensity was measured using the quantification analysis tool that is available in the Leica software (Leica LAS-AF Software).

To track the intracellular location of NPs, MDA-MB-453 cell monolayer was incubated with c6 loaded NPs for 6 h. Cells were washed three times with PBS and mitochondria were stained with MitoTracker Red CMXRos (Life Technologies) for 30 min at $50 \times 10^{-9}\text{ M}$, and then washed three times with PBS. Cells were fixed using a paraformaldehyde solution in PBS (3.7% w/v) for 15 min and observed by CLSM. This work has been performed by the Confocal Microscopy Service of the Universidad de Alcalá de Henares (UAH) and the Biomedical Networking Center (CIBER-BBN), located at the facilities of the Cell Culture Unit.

3.5.3. In Vitro Cytotoxicity Assay—The cytotoxicity of diverse concentrations of NPs (2.5, 1.25, 0.63, 0.31, and 0.16 mg mL⁻¹) was analyzed by Alamar Blue assay (Sigma-Aldrich) in different cell cultures.^[60] For this propose, MCF-7, MDA-MB-453, or HMEpiC were seeded at 30 000 cells mL⁻¹ (15 000 cells per well) in 24-well plate, 35 000 cells mL⁻¹ (20 500 cells per well) in 24-well plates or 80 000 cells mL⁻¹ (40 000 cells per well) in 48-well plates, respectively.

After 24 h of incubation, the medium was replaced with the NPs dispersed in NaCl (50:50 v/v of the NPs suspension and completed medium). The plates were incubated at 37 °C in a humidified air with 5% CO₂ for 24 h. Then, 100 µL of Alamar Blue solution (10% Alamar Blue solution in phenol red free DMEM medium) was added to all wells and the fluorescence was measured on a Biotek SYNERGY-HT plate reader ($\lambda_{\text{excitation}} = 530$ nm and $\lambda_{\text{emission}} = 590$ nm) after 4 h of incubation.

3.5.3.1. Statistical Analysis: Results were expressed as mean $\pm$ standard deviation. Statistical significance (significance level of: * $p < 0.05$) was evaluated using the analysis of variance (ANOVA, Tukey test) as required, by Origin 9.

4. Conclusions

The chemical incorporation of small amounts of HEMA in the hydrophobic block of PEG-*b*-polyMTOS (obtained by RAFT polymerization), to give rise to PEG-*b*-(poly(MTOS-*co*-HEMA) terpolymers, significantly increased the bioactivity of the correspondent NPs (NP-10H). HEMA reduced the hydrophobicity of the polyMTOS block without jeopardizing NP self-assembly as the hydrodynamic diameter and polydispersity were maintained or even improved if compared with the NPs obtained with no HEMA (PEG-*b*-polyMTOS). The terpolymers were also able to encapsulate additional α -TOS (NP-10H+ α -TOS) with high efficiency and the resulting NPs presented the highest bioactivity with the highest selectivity. These results indicate that the strategy followed in this work increased dramatically the biological activity of α -TOS based NPs, probably by improving the water accessibility to the MTOS residues and favoring its hydrolysis and the release of the anticancer compound.

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Figure 2. Comparison of ATR-FTIR spectra of MTOS (red), CTA-PEG₈ (blue), PEG-10H (gray), and PEG-20H (green).

Figure 3.

Kinetics of polymerization of A) PEG-10H and B) PEG-20H, including the variation of $\ln([M]_0/[M]_t)$ as a function of reaction time and M_n , and polydispersity as a function of MTOS conversion. Normalized SEC traces during the polymerization of C) PEG-10H and D) PEG-20H.

Figure 4.

A) Particle size distributions (D_h , by intensity) of unloaded NP, measured by DLS. Red: NP-10H; blue: NP-20H. B) SEM micrographs of NP-10H polymeric system dried from aqueous solution at room temperature. C,E) Particle size distributions of α -TOS entrapped NPs. Black: NP-10H+ α -TOS; orange: NP-20H+ α -TOS; green: NP-87+ α -TOS, and purple: NP-71+ α -TOS. SEM micrographs of D) NP-10H+ α -TOS and F) NP-87+ α -TOS dried from aqueous solution at room temperature.

Figure 5.

A) Monitoring of endocytosis of c6 loaded NP-10H, including confocal micrographs of MDA-MB-453 cells after 0.5, 2, and 6 h of NPs incubation. Colocalization of c6 loaded NP-10H B) and NP-20H C) into the mitochondria of MDA-MB-453 cells by confocal microscopy.

Figure 6.

MDA-MB-453 viability in the presence of different α -TOS entrapped NPs concentrations, measured after 24 h with respect to the control. Cell viability of unloaded NPs is also shown for all copolymer systems. A) Red: NP-71 $\pm\alpha$ -TOS, B) green: NP-76 $\pm\alpha$ -TOS, C) gray: NP-82 $\pm\alpha$ -TOS, and D) blue: NP-87 $\pm\alpha$ -TOS. The diagrams include the mean, the standard deviation ($n = 8$), and the ANOVA results at a significance level of $*p < 0.05$.

Figure 7. MCF-7 viability in the presence of unloaded and α -TOS-loaded NP-71 at 2.5 mg mL^{-1} measured overtime until 72 h. The diagram includes the mean, the standard deviation ($n = 8$), and the ANOVA results with respect to control at a significance level of: * $p < 0.05$.

Figure 8.

A) Comparison of MCF-7 viability in the presence of unmodified NP-71 and NP-10H and NP-20H formulation at 2.5 mg mL⁻¹, measured overtime until 72 h. B) Comparison of MDA-MB-453 viability in the presence of different concentrations of unloaded and α -TOS loaded NP-10H, measured after 24 h. The diagram includes the mean, the standard deviation ($n = 8$), and the ANOVA results with respect to control at a significance level of $*p < 0.05$.

Figure 9.

HMEpiC (blue line) and MDA-MB-453 (red line) cell viability in the presence of different concentrations of NPs measured after 24. A) NP-71, B) NP-10H, C) NP-71+ α -TOS, and D) NP-10H+ α -TOS. The diagrams include the mean, the standard deviation ($n = 8$), and the ANOVA results from the comparison of cancer cells and healthy cells, obtained at a significance level of $\#$, $*p < 0.05$.

Table 1

Experimental conditions and the most relevant structural characteristics of PEG-*b*-poly(MTOS-*co*-HEMA) amphiphilic copolymers.

Sample	[MTOS]: [HEMA][mol%]	[Metha]:[CTA]: [AIBN][mol%]	X_{MTOS} [%]	X_{HEMA} [%]	$M_n \times 10^{-3a)}$	$\bar{P}^d)$	$n_{\text{PEG}}^b)$	$m_{\text{MTOS}}^b)$	$F_{\text{PEG}}-F_{\text{Metha}}$ [mol%]	$T_{\text{max}}^c)$ [°C]
PEG-10H	90:10	[100]:[1]:[0.3]	89	100	51.6	1.26	177	51	76–24	405
PEG-20H	80:20	[100]:[1]:[0.3]	82	100	47.8	1.25	177	35	80–20	407

^{a)}Determined by SEC (THF) using PMMA standards

^{b)}Determined using ¹H-NMR spectroscopy

^{c)}Measured by TGA under nitrogen atmosphere.

Table 2

Most relevant characteristics of unloaded based on PEG-10H and PEG-20H and loaded NPs: hydrodynamic diameter (D_h , by intensity), size distribution width (W_d), polydispersity index (PDI), and zeta potential values (ζ), measured by dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE), respectively.

NP sample	EE [%]	D_h [nm]	W_d [nm]	PDI	ζ [mV]
NP-0	–	134.9 ± 6.5	31	0.079 ± 0.012	–2.38
NP-10H	–	104.2 ± 10.3	24	0.061 ± 0.002	–2.36
NP-20H	–	144.0 ± 8.2	35	0.057 ± 0.010	–1.60
NP-10H+α6	89.8	115.8 ± 6.5	52	0.098 ± 0.018	–1.86
NP-20H+α6	90.1	160.3 ± 7.7	46	0.106 ± 0.021	–0.98
NP-10H+α-TOS	75.1	123.6 ± 9.2	45	0.114 ± 0.059	–2.01
NP-20H+α-TOS	88.4	155.2 ± 8.3	47	0.086 ± 0.019	–1.94
NP-71+α-TOS	88.5	153.6 ± 7.6	39	0.122 ± 0.034	–2.26
NP-76+α-TOS	94.9	140.3 ± 6.6	60	0.127 ± 0.013	–1.84
NP-82+α-TOS	98.6	145.1 ± 5.2	44	0.075 ± 0.010	–1.29
NP-87+α-TOS	95.8	95.9 ± 2.3	21	0.096 ± 0.010	–1.01