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Synthesis of chemically functionalized superparamagnetic nanoparticles as delivery vectors for chemotherapeutic drugs

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Abstract—Superparamagnetic iron oxide nanoparticles (SPIONs) are in clinical use for disease detection by MRI. A major advancement would be to link therapeutic drugs to SPIONs in order to achieve targeted drug delivery combined with detection. In the present work, we studied the possibility of developing a versatile synthesis protocol to hierarchically construct drug-functionalized-SPIONs as potential anti-cancer agents. Our model biocompatible SPIONs consisted of an iron oxide core (9–10 nm diameter) coated with polyvinylalcohols (PVA/aminoPVA), which can be internalized by cancer cells, depending on the positive charges at their surface. To develop drug-functionalized-aminoPVA-SPIONs as vectors for drug delivery, we first designed and synthesized bifunctional linkers of varied length and chemical composition to which the anti-cancer drugs 5-fluorouridine or doxorubicin were attached as biologically labile esters or peptides, respectively. These functionalized linkers were in turn coupled to aminoPVA by amide linkages before preparing the drug-functionalized-SPIONs that were characterized and evaluated as anti-cancer agents using human melanoma cells in culture. The 5-fluorouridine-SPIONs with an optimized ester linker were taken up by cells and proved to be efficient anti-tumor agents. While the doxorubicin-SPIONs linked with a Gly-Phe-Leu-Gly tetrapeptide were cleaved by lysosomal enzymes, they exhibited poor uptake by human melanoma cells in culture.

1. Introduction

Drug entrapping polymeric nanoparticles have been well studied, and some are under clinical evaluation for the treatment of cancer.[1–3] The nanoparticles can act as drug vectors in matrix systems where drugs are either incorporated into a hydrophobic or hydrophilic core surrounded by a polymeric membrane, or displayed at the nanoparticle surface. The release of these drugs from the nanoparticles may either be a passive phenomenon,[3,4] which depends on the properties of the polymers and the cellular environment (ionic strength, pH, redox state), or an active phenomenon,[5–7] where in isolated instances drugs have been covalently linked to the polymeric nanocarriers via enzyme-specific releasing routes. Anti-cancer drugs non-covalently bound to superparamagnetic iron oxide nanoparticles (SPIONs) have been studied as potential targeted drug carriers with some promise.[8–16] A less studied area is to covalently conjugate[17–21] an anti-tumor agent to SPIONs so that they can be internalized by the cancer cells, and the drug subsequently released through an enzyme mediated mechanism to exert its expected therapeutic effects with minimal toxicity. With this ultimate objective in mind, we first set out to covalently attach two well known anti-tumor agents, 5-fluorouridine (Fur) and doxorubicin (DOX), to a mixed polymer of polyvinylalcohol (PVA) and poly(vinylalcohol/vinylamine) (aminoPVA), then to construct the drug-SPION conjugate by adding the ferrofluid consisting of iron oxide nanoparticles. The aim of the present study was to develop chemical methods of ligating the drugs to the aminoPVA polymer through appropriate bifunctional linkers capable of releasing the active entity intracellularly. In previous studies, we have prepared and characterized various SPIONs coated with polyvinylalcohols, which were found to be internalized by non-phagocytic human tumor cells.[22–24] We report herein that a hierarchical assembly of drug-SPION conjugates is chemically feasi-

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ble, and that internalization by human melanoma cancer cells affects their survival.

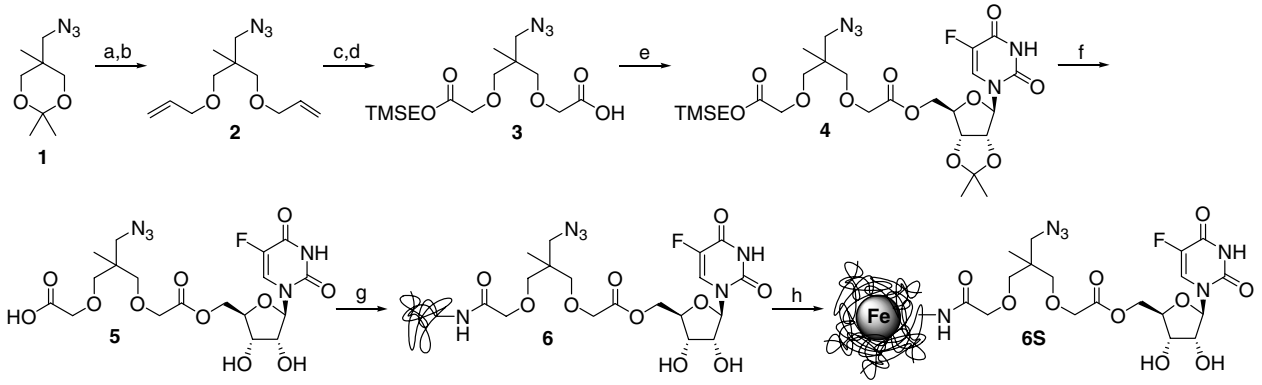
2. Chemistry

2.1. Synthesis of drug-functionalized-aminoPVAs

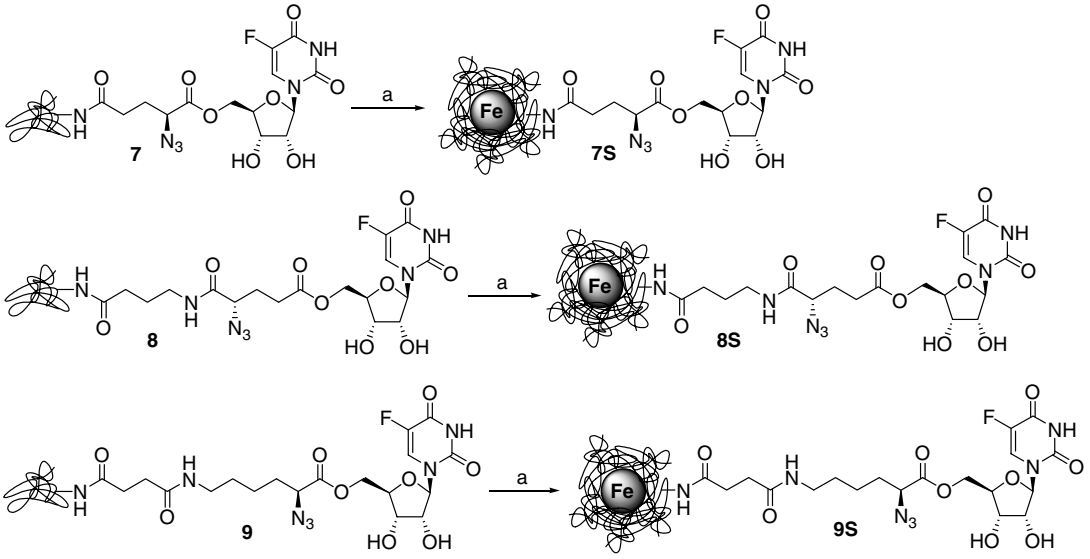
In order to evaluate the optimal ester linkage we chose 5-fluorouridine 5′-esters as a prototypical anti-tumor agent, which would be released intracellularly by the action of esterases. We varied the length of the dicarboxylic acid tethers, each containing an azide group as an IR probe to assess the extent of conjugation (loading) with the aminoPVA (Supplemental Table S1).

The preferred linker consisted of an end-differentiated azido dicarboxylic acid as shown in Scheme 1. The readily available acetonide **1**[25] was converted into the bis-allyl ether **2**, and the latter was oxidized[26] and selectively protected as the monoester **3**. Esterification with 2′,3′-isopropylidene-5-fluorouridine[27] gave the ester **4**, which was subsequently converted to the free acid **5**. Amide formation with the aminoPVA was realized using EDC and HOBt in aqueous DMF. Purification by dialysis and lyophilization gave a white amorphous solid **6** where 43% of the amines on aminoPVA were conjugated with 5-fluorouridine as determined by IR analysis. The level of drug incorporation was determined by measuring the ratio of the area under the azide peak at 2100 cm^{-1} to that of a reference peak in the aminoPVA at 1100 cm^{-1} in the IR spectrum. Treatment of **5** in aqueous buffer with pig liver esterase at pH 7.2 resulted in the release of 5-fluorouridine within 24 h, while **6** released about 50% of the 5-fluorouridine under the same conditions. The drug-linker conjugate was stable in the pH range 5–7 for several days. Three additional linkers were also prepared and coupled to aminoPVA to give 5-fluorouridine-linked aminoPVAs **7**, **8**, and **9** containing 27%, 22%, and 19% of 5-fluorouridine, respectively (Scheme 2 and Supplementary Information).

Preparation of the DOX-linker-aminoPVA conjugate is shown in Scheme 3. We used a Gly-Phe-Leu-Gly tetrapeptide linker known to be cleaved by the lysosomal thiol protease cathepsin B.[28] Standard peptide chemistry



Scheme 1. Reagents and conditions: (a) cat. HCl, THF/H_2O, 90 °C, 2 h; (b) NaH, THF, 15 min, then allyl bromide, 24 h, rt; (c) $NaIO_4$, $RuCl_3{\cdot}H_2O$, CCl_4/MeCN/H_2O (1:1:1.5), rt, o/n; (d) 2-(trimethylsilyl)ethanol, EDC, DMAP, CH_2Cl_2, rt, o/n; (e) 2′,3′-isopropylidene-5-fluorouridine, EDC, DMAP, CH_2Cl_2, rt, 2 d; (f) TFA/H_2O (11:1), rt, 35 min; (g) EDC, HOBt, aminoPVA, DMF/H_2O, rt, 1 d; (h) ferrofluid.



Scheme 2. Assembly of 5-fluorouridine-SPIONs **7S**, **8S**, and **9S**. Reagents: (a) ferrofluid.

starting with the trimethylsilylethyl ester of *N*-Cbz-Gly **10** led to the protected peptide **13**. A 2-azido-L-Glu moiety[29] was appended to furnish the Fm ester **14**, which was coupled from the Gly OH end to DOX giving **15** after deprotection of the ester. Coupling with aminoPVA using EDC/HOBT in aqueous DMF, and purification by multiple cycles of dialysis gave the adduct **16** as a red solid after lyophilization. Quantitative IR analysis estimated that 28% of the amines on aminoPVA were functionalized with **15**.

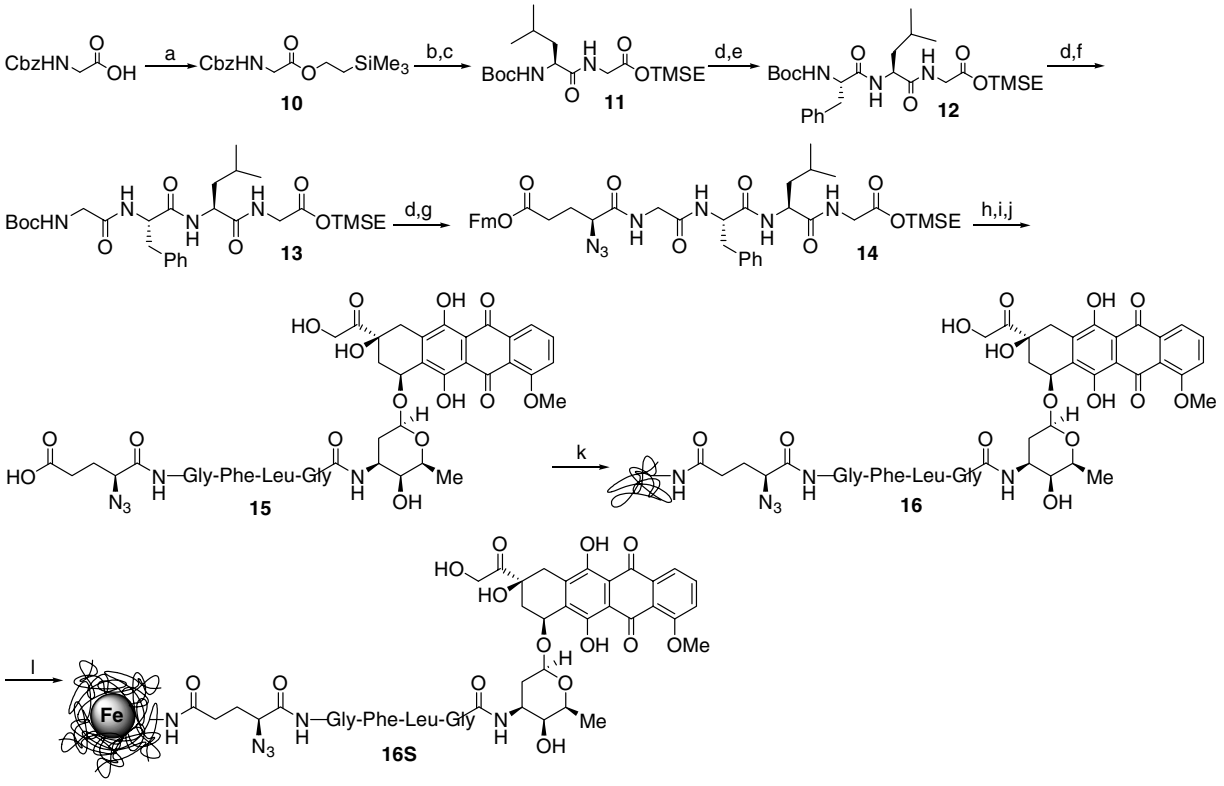
2.2. Preparation and characterization of drug-SPION adducts

The drug-functionalized-aminoPVA-SPIONs (drug-SPIONs) were prepared by adding the ferrofluid to mixtures of PVA/aminoPVA and drug-linker-aminoPVA to obtain homogeneous amber-brown colored solutions. We have previously shown that SPIONs coated with PVA/aminoPVA at a ratio of 45:1 (w/w) and a polymer/iron ratio of 10:1 (w/w) are efficiently taken up by cells,[23,24] and that the efficiency and biocompatibility of the uptake were dependent on the number of positive charges at the surface of aminoPVA-SPIONs.[23] The number of positive charges was maintained by compensating for the positive charges lost through drug substitution of the amino groups on the aminoPVA. This was accomplished by increasing the ratio of aminoPVA to PVA during coating of the ferrofluid (Supplemental Table S2). A constant ratio of polymer to iron of 10 was maintained.

The drug-SPIONs were characterized for their size and surface charge by photon correlation spectroscopy (PCS) (Table 1) and were consistent with their expected compositions. All drug-SPIONs and aminoPVA-SPIONs had comparable size and particle size distributions as measured by PCS. Electrophoretic mobility (charge-to-size ratio) and zeta potential were calculated according to the Smoluchowski approximation.

To evaluate the stability of the drug-SPIONs at physiological pH, we performed gel-filtration experiments on the 5-fluorouridine-SPIONs-1 **6S** and DOX-SPIONs **16S** at pH 7, and compared the elution profile of iron and PVA for 5-fluorouridine-SPIONs (Fig. 1A) and iron and PVA elution as well as the fluorescence of DOX for DOX-SPIONs (Fig. 1B). The 5-fluorouridine-SPIONs **6S** and DOX-SPIONs **16S** eluted as a single high-molecular weight fraction encompassing iron, PVA, and the fluorescent DOX, with negligible elution of low-molecular weight fluorescent molecules.

In order to stabilize the various SPIONs for storage, an excess of PVA has to be added during the preparation[22] and some trailing can be observed after the high-molec-



Scheme 3. Reagents and conditions: (a) 2-(trimethylsilyl)ethanol, EDC, DMAP, CH_2Cl_2, rt, 24 h; (b) H_2, 10% Pd/C, 40 psi, rt, 16 h; (c) $BocLeuCO_2H{\cdot}H_2O$, EDC, HOBt, *i*-Pr_2EtN, DMF, 0 °C, 1 h, 0 °C → rt, o/n; (d) 4 N HCl/dioxane, rt, 1 h; (e) $BocPheCO_2H$, EDC, HOBt, *N*-methylmorpholine, DMF, 0 °C → rt, 1 d; (f) $BocGlyCO_2H$, EDC, HOBt, *N*-methylmorpholine, DMF, 0 °C → rt, 1 d; (g) 2-azido-pentanedioic acid 5-(9*H*-fluoren-9-ylmethyl) ester, EDC, HOBt, *N*-methylmorpholine, DMF, 0 °C → rt, 1 d; (h) TFA/CH_2Cl_2 1:1, rt, 80 min; (i) EDC, HOBt, DMF, rt, 30 min, then doxorubicin·HCl, NEt_3, rt, 1 d; (j) 1:2 *N*-methylpyrrolidine/DMF, rt, 1 h; (k) EDC, HOBt, NEt_3, aminoPVA, DMF/H_2O, rt, 1 d; (l) ferrofluid.

Table 1. Physicochemical characteristics of the drug-SPIONs prepared

Coated nanoparticles	Nanoparticle diameter [nm]	pH	Calculated free amine [mM]	Mobility [$10^{-8}ms^{-1}V^{-1}m$]	Zeta potential [mV]
AminoPVA-SPIONs	56.4 ± 4.1	7.00	1.59	1.3 ± 0.1	17.0 ± 0.5
Fur-SPIONs-1 **6S**	67.2 ± 1.3	6.85	1.59	1.2 ± 0.1	14.8 ± 1.2
Fur-SPIONs-2 **7S**	74.7 ± 6.7	6.64	1.59	1.4 ± 0.1	18.0 ± 0.7
Fur-SPIONs-3 **8S**	52.6 ± 6.5	6.30	1.59	1.2 ± 0.1	14.8 ± 0.8
Fur-SPIONs-4 **9S**	113.7 ± 16.3	6.82	1.59	1.2 ± 0.1	15.4 ± 0.8
DOX-SPIONs **16S**	21.6 ± 2.5	6.33	1.61	1.5 ± 0.1	19.2 ± 0.6

The size, pH, surface charge (amino groups), and the electrophoretic mobility and zeta potential are provided. Residual free amino groups on the aminoPVA were calculated from the known number (2.5% N, w/w) of amino groups in unfunctionalized-aminoPVA and the number of drugs per aminoPVA.

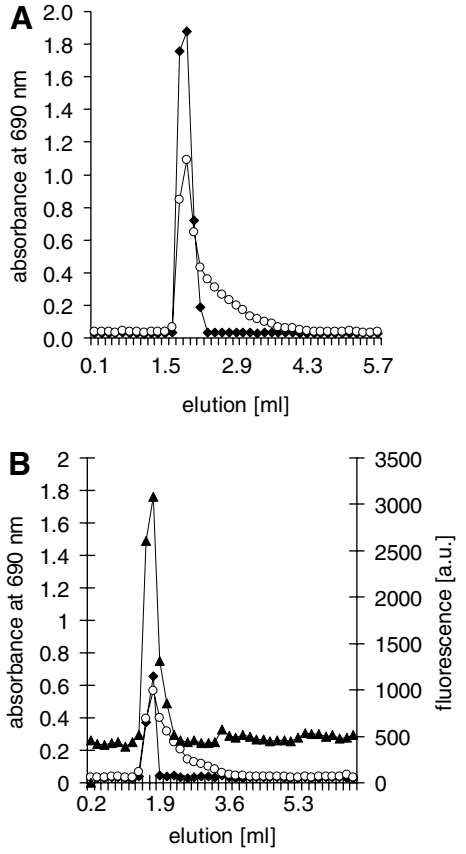


Figure 1. Gel-filtration of the drug-SPIONs. Fur-SPIONs-1 **6S** (A) or DOX-SPIONs **16S** (B) were gel chromatographed on Sephadex G-75 in phosphate buffer at pH 7. Iron (♦) and PVA (○) were measured in the eluting fractions at 690 nm by Prussian Blue and iodine reactions, respectively. SPION-bound doxorubicin (▲) was determined by its fluorescence at $\lambda_{ex}/\lambda_{em}$ 485/580 nm.

ular weight peak of the gel-filtration. However, these trailing fractions did not display anti-cancer activity when evaluated on cells (results not shown).

3. Biological results

3.1. Effects of 5-fluorouridine-SPIONs on human melanoma cells

Uptake by human Me300 melanoma cells of 5-fluorouridine-SPIONs1–4 **6S**, **7S**, **8S**, and **9S** was determined by the amount of cell-bound iron (Fig. 2A). With the exception of **7S**, all drug-SPIONs were taken up by Me300 cells, as compared with the uptake of the previously described aminoPVA-SPION.[23–25] In order to better understand the physicochemical characteristics of the linker allowing for optimal cell uptake, we compared the calculated length and hydrophobicity of the 5-fluorouridine-linker conjugates with their actual uptake by melanoma cells (Table 2). The rate of cell uptake of 5-fluorouridine-SPIONs was dependent on the length of the particular linker, but not its hydrophobicity (Table 2). The highest uptake by Me300 cells was exhibited by **6S**. A time-dependent effect on DNA synthesis and cell survival was observed (Fig. 2C). The 5-fluorouridine-SPIONs **6S** acted more slowly than free 5-fluorouridine (Fig. 2B), but when exposure time of cells to **6S** was increased, we observed an efficient inhibition of melanoma cell survival and DNA synthesis, demonstrating the potential of drug-ester functionalization of SPIONs in drug delivery. It should be noted that although active as an anti-tumor agent, 5-fluorouridine itself is not used in the clinic due to side-effects such as leucopenia, thrombopenia, and gastrointestinal toxicity.[30]

3.2. Effects of DOX-SPIONs on human melanoma cells

Uptake by human Me300 melanoma cells of DOX-SPIONs **16S** was evaluated by the amount of cell-bound iron and fluorescence (Fig. 3A), as compared with that of the previously described aminoPVA-SPIONs.[22–24] The uptake of DOX-SPIONs by Me300 melanoma cells was much lower than that of aminoPVA-SPIONs, even if the positive cell surface charges necessary for cell uptake were maintained at the same level in the DOX-SPIONs as in the aminoPVA-SPIONs (Table 1). No decrease in either DNA synthesis or cell survival was observed with DOX-SPIONs **16S**, while free DOX as a control was highly active in these cells (Fig. 3B). In order to determine whether proteases from melanoma cells have the potential to release DOX from the DOX-SPIONs **16S**, we extracted lysosomal enzymes from the melanoma cells under conditions able to maintain thiol-cathepsin activity,[31] and exposed DOX-SPIONs **16S** to this extract. Release of free DOX was demonstrated by gel-filtration, showing the potential of lysosomal enzymes under our cell extraction conditions to release DOX from our DOX-SPIONs **16S** (Fig. 3C), and suggesting that the DOX-SPIONs can be suitable substrates for these lysosomal proteases. It is possible that the amount of DOX-SPIONs **16S** taken up by Me300 melanoma cells is too low to achieve efficacy.

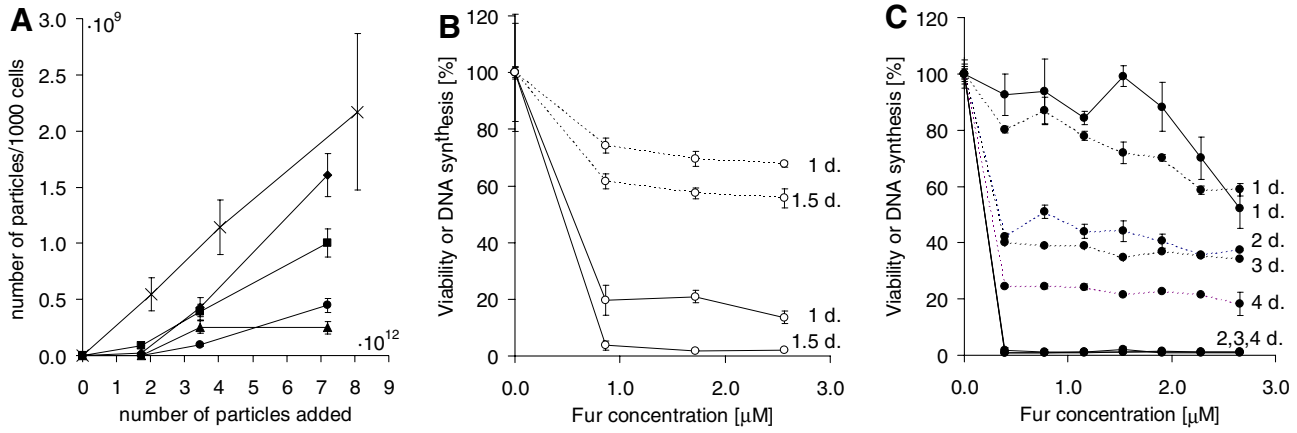


Figure 2. Uptake and cellular effects of the Fur-SPIONs **6S**–**9S** in human melanoma cells. (A) Human Me300 melanoma cells were exposed for 16 h to increasing concentrations of the Fur-SPIONs **6S** (●), **7S** (▲), **8S** (♦) or **9S** (■), or aminoPVA-SPIONs (**X**) for comparison, and the cell uptake of the Fur-SPIONs was determined by cell-bound iron content. (B) Human Me300 melanoma cells were exposed to increasing concentrations of free Fur then either cellular metabolic activity (Alamar Blue reduction, (●) dotted lines) or DNA synthesis ([^{3}H]thymidine incorporation, (•) solid lines) was determined. (C) Human Me300 melanoma cells were exposed for 1–4 days (1d–4d) to Fur-SPIONs-1 **6S**, then either cellular metabolic activity (Alamar Blue reduction, (●) dotted lines) or DNA synthesis ([^{3}H]thymidine incorporation, (●) solid lines) was determined.

Table 2. Relationship between the length or hydrophobicity of the linker-Fur with uptake of the various Fur-SPIONs by human Me300 melanoma cells

Drug-SPION	% Iron uptake at 16 h	Linker length [Å]	log P[a]
AminoPVA-SPIONs	41 ± 12	—	—
Fur-SPIONs-1 **6S**	37 ± 4	18.95	−1.165
Fur-SPIONs-2 **7S**	5 ± 1	13.23	−1.220
Fur-SPIONs-3 **8S**	32 ± 1	20.65	−1.881
Fur-SPIONs-4 **9S**	24 ± 2	22.18	−1.067
5-Fluorouridine	—	—	−1.676

Cells were exposed for 16 h to the Fur-SPIONs **6S**, **7S**, **8S** or **9S**, then the percentage of SPIONs taken up by cells was determined as the ratio (%) of cell-bound iron to the amount of iron added to the cells. This uptake was compared to the calculated length or the hydophobicity (log P) of the different linkers.

[a] log P was calculated from www.molinspiration.com/cgi-bin/properties.

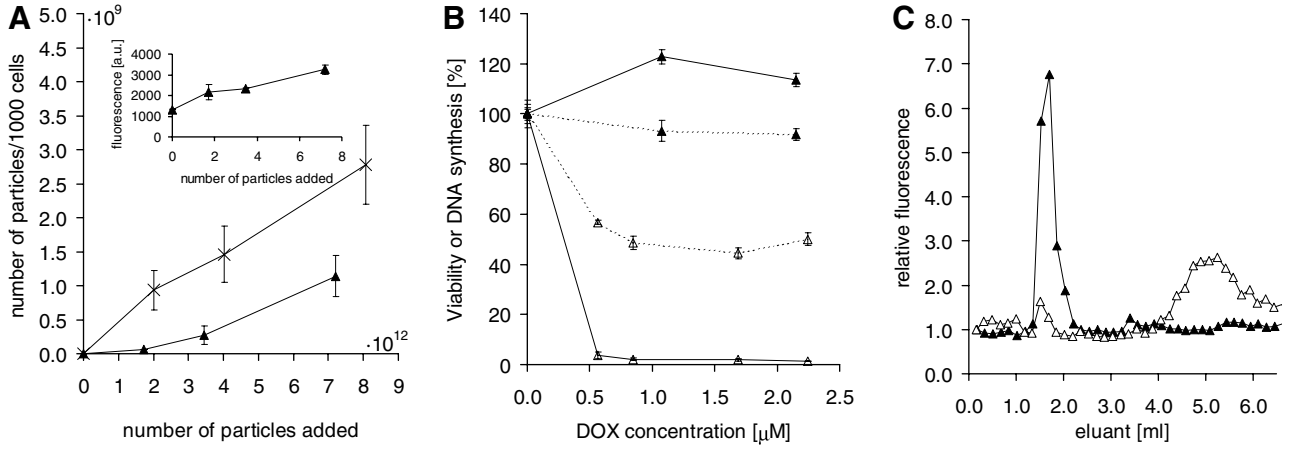


Figure 3. Uptake and cellular effects of the DOX-SPIONs **16S** in human melanoma cells. (A) Human Me300 melanoma cells were exposed for 36 h to increasing concentrations of DOX-SPIONs **16S**. The uptake of DOX-SPIONs **16S** was determined by measuring cell-bound iron (▲) and the fluorescence of cell-bound DOX (insert). The uptake of aminoPVA-SPIONs (**X**) was determined for comparison. (B) Human Me300 melanoma cells were exposed for 36 h to increasing concentrations of either free DOX or DOX-SPIONs **16S**, then either cell survival (Alamar Blue reduction, AlaB) or DNA synthesis ([^{3}H]thymidine incorporation, ^{3}HT) was determined as the ratio of treated to untreated cells. Free DOX (AlaB: △, dotted line; ^{3}HT: △, solid line); DOX-SPIONs **16S** (AlaB: ▲, dotted line; ^{3}HT: ▲, solid line). (C) Either native DOX-SPIONs **16S** (▲) or DOX-SPIONs **16S** exposed for 24 h to proteases extracted from human Me300 melanoma cells (△) were chromatographed on Sephadex G-75 in phosphate buffer at pH 7. DOX fluorescence of either free DOX or SPIONs-bound DOX was measured in the eluting fractions at $\lambda_{ex}/\lambda_{em}$ 485/580 nm as the relative fluorescence to the baseline.

4. Conclusion

In conclusion, we have developed a versatile chemical synthesis protocol allowing for the hierarchical construction of covalent drug-SPION conjugates with mixed carrier polymers, such as PVA and aminoPVA. We have also shown that for doxorubicin and 5-fluorouridine, the cell uptake mechanisms, the accessibility of enzymatic activities for releasing the drugs from their SPIONs carriers, in particular the length and chemical structure of the linker, and the intracellular routing of these drug-SPIONs are important parameters for eventual drug delivery. Further studies evaluating the subcellular localization of the various drug-functionalized-SPIONs are underway. The survival of melanoma cells was most significantly reduced with the 5-fluorouridine-SPION conjugate **6S** resulting in decreased melanoma cell viability and DNA synthesis ($IC_{50} < 0.5$ μM) after 48 h exposure. Further studies with other anti-tumor agents conjugated to SPIONs as ester linkages are underway in our laboratories and will be published in due time.

5. Experimental

5.1. Chemistry

5.1.1. General. All commercially available reagents were used without further purification. All reactions were performed under nitrogen atmosphere. NMR (^1H, ^{13}C) spectra were recorded on Bruker ARX-400, AV-300, and AV-400 RG spectrometers. Low- and high-resolution mass spectra were recorded using electrospray technique. Optical rotations were recorded on a Perkin-Elmer Model 343 polarimeter in a 1 dm cell at ambient temperature. Analytical thin-layer chromatography was performed on pre-coated silica gel plates. Flash column chromatography was performed using (40–63 μm) silica gel. IR was measured using Perkin-Elmer Spectrum One FT-IR spectrometer. IR spectra of organic compounds were taken as film, while of polymer mixtures they were taken after aqueous polymer sample was mixed with KBr and lyophilized to give a fine powder, which was pressed into a disk.

5.1.2. 3-(3-Allyloxy-2-azidomethyl-2-methyl-propoxy)-propene (2). To a solution of **1**[25] (3.26 g, 17.6 mmol) in THF (59 mL) and H_2O (12 mL) were added 40 drops of concentrated HCl and the mixture was stirred at 90 °C for 2 h. The solvent was removed in vacuo and to remove residual water, the crude product was dissolved in EtOAc, dried ($MgSO_4$), and concentrated to give the diol[25,32] as a white solid (2.55 g, quant); mp 40–42 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 3.59 (4H, s), 3.44 (2H, s), 2.81 (2H, s), 0.85 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 68.0, 55.6, 41.0, 17.6; IR (neat/NaCl) 3351, 2936, 2104, 1468, 1283, 1042 cm^{-1}; LRMS (ESI, *m*/*z*, MH^+) 146. To a solution of the diol (9.73 g, 67.0 mmol) in THF (335 mL) was added sodium hydride (60% suspension in oil, 8.04 g, 201.1 mmol). After stirring the mixture at room temperature for 15 min, allyl bromide (24.33 g, 201.1 mmol) was added and reaction mixture was stirred for 24 h. After evaporation of DMF, the residue was dissolved in H_2O and the aqueous layer was extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$), and concentrated. Product was obtained following column chromatography (2.5:97.5 EtOAc/hexane) as a yellow oil (14.3 g, 95%); ^1H NMR (400 MHz, $CDCl_3$) δ 5.94–5.84 (2H, m), 5.29–5.15 (4H, m), 3.97 (2H, t, $J = 1.5$Hz), 3.95 (2H, t, $J = 1.5$ Hz), 3.32 (2H, s), 3.28 (2H, s), 3.27 (2H, s), 0.98 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.0, 117.0, 73.1, 72.4, 55.7, 41.2, 18.3; IR (neat/NaCl) 2857, 2103, 1273, 1093 cm^{-1}.

5.1.3. [2-Azidomethyl-2-methyl-3-(2-trimethylsilanyl-ethoxycarbonylmethoxy)-propoxy]-acetic acid (3). To a solution of **2** (1.05 g, 4.66 mmol) in CCl_4/MeCN/H_2O 1:1:1.5 (56 mL) were added sequentially sodium periodate (8.17 g, 38.2 mmol) and ruthenium trichloride monohydrate (0.043 g, 0.20 mmol) and the thick mixture was stirred vigorously at room temperature overnight. The reaction mixture was diluted with H_2O and CH_2Cl_2 until the solids dissolved and the aqueous layer was extracted with CH_2Cl_2 (4×), dried ($MgSO_4$) and concentrated. The resulting brown oil was dissolved in saturated $NaHCO_3$ and washed twice with EtOAc. The aqueous layer was acidified with 6 N HCl and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated to give the biscarboxylic acid as a yellow-brown solid, which was used without further purification (0.920 g, 76%); ^1H NMR (300 MHz, $CDCl_3$) δ 10.34 (2H, s), 4.14 (4H, s), 3.45 (4H, s), 3.37 (2H, s), 0.99 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.6, 74.5, 68.3, 55.5, 41.0, 17.9; IR (neat/NaCl) 3117, 2920, 2105, 1729, 1246, 1131 cm^{-1}; LRMS (ESI, *m*/*z*, MH^+) 262. The crude biscarboxylic acid (0.500 g, 1.91 mmol) was dissolved in CH_2Cl_2 (15 mL) and to it were added 2-(trimethylsilyl)ethanol (274 μL, 1.91 mmol), DMAP (0.117 g, 0.96 mmol), and EDC (0.367 g, 1.91 mmol). The mixture was stirred at room temperature for 16 h. The crude reaction was diluted with H_2O and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (0:10–1:9 MeOH/EtOAc) as a yellow oil (0.256 g, 37%); ^1H NMR (300 MHz, $CDCl_3$) δ 10.20 (1H, s), 4.24–4.19 (2H, m), 4.06 (2H, s), 4.03 (2H, s), 3.42–3.34 (6H, m), 1.01–0.96 (5H, m), 0.02 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.8, 170.8, 74.6, 74.4, 69.0, 68.7, 63.4, 55.4, 40.9, 17.9, 17.4, −1.5; IR (neat/NaCl) 3204, 2955, 2103, 1751, 1132, 838 cm^{-1}; LRMS (ESI, *m*/*z*, MH^+) 362.

5.1.4. {2-Azidomethyl-3-[6-(5-fluoro-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-ylmethoxycarbonylmethoxy]-2-methyl-propoxy}-acetic acid 2-trimethylsilanyl-ethyl ester (4). To a solution of **3** (0.275 g, 0.76 mmol) in CH_2Cl_2 (5 mL) were added DMAP (0.046 g, 0.38 mmol) and 2′,3′-isopropylidene-5-fluorouridine[27] (0.253 g, 0.84 mmol). Lastly EDC (0.160 g, 0.84 mmol) was added and reaction mixture was stirred at room temperature for 2 days. The crude reaction mixture was diluted with H_2O and extracted with CH_2Cl_2 (4×). The combined organic lay-

ers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (1:1 hexane/EtOAc) as a pale yellow oil (0.260 g, 54%); ^1H NMR (400 MHz, $CDCl_3$) δ 9.94 (1H, d, J = 4.7 Hz), 7.42 (1H, d, J = 5.7 Hz), 5.65 (1H, d, J = 1.8 Hz), 4.97 (1H, dd, J = 1.9 Hz, J = 6.4 Hz), 4.80 (1H, dd, J = 3.5 Hz, J = 6.3 Hz), 4.40–4.32 (3H, m), 4.21–4.17 (2H, m), 4.10–4.00 (4H, m), 3.40–3.33 (6H, m), 1.53 (3H, s), 1.32 (3H, s), 0.99–0.95 (5H, m), 0.01 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 170.0, 157.0 (d, J = 26.6 Hz), 148.8, 140.6 (d, J = 238.9 Hz), 126.5 (d, J = 33.8 Hz), 114.9, 94.6, 85.1, 84.4, 80.8, 74.5, 74.3, 68.8, 68.6, 64.0, 63.3, 55.4, 41.1, 27.2, 25.3, 17.8, 17.4, −1.4; IR (neat/NaCl) 3215, 2955, 2104, 1712, 1465, 1252, 1132 cm^{-1}; LRMS (ESI, m/z, MH^+) 646.

5.1.5. {2-Azidomethyl-3-[5-(5-fluoro-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-3,4-dihydroxy-tetrahydro-furan-2-ylmethoxycarbonylmethoxy]-2-methyl-propoxy}-acetic acid (5). The ester **4** (0.072 g, 0.11 mmol) was dissolved in TFA/H_2O 11:1 (4.3 mL) and stirred at room temperature for 35 min. Solvent was removed in vacuo and then traces of TFA were removed by co-evaporating with MeOH. The product was obtained after column chromatography (95:4:1 EtOAc/MeOH/AcOH) as an amorphous solid (0.032 g, 56%); ^1H NMR (300 MHz, CD_3OD) δ 7.86 (1H, d, J = 6.7 Hz), 5.81 (1H, dd, J = 1.3 Hz, J = 3.8 Hz), 4.44 (2H, d, J = 3.6 Hz), 4.21–4.10 (5H, m), 4.05 (2H, s), 3.44 (2H, s), 3.40 (2H, s), 3.37 (2H, s), 0.98 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 174.0, 171.7, 159.4 (d, J = 26.2 Hz), 150.8, 141.8 (d, J = 233.4 Hz), 126.1 (d, J = 34.7 Hz), 91.7, 82.9, 75.4, 75.1, 75.0, 71.0, 69.6, 69.3, 64.6, 56.5, 42.1, 18.1; IR (neat/NaCl) 3404, 2105, 1709, 1259, 1125 cm^{-1}; LRMS (ESI, m/z, MH^+) 506; HRMS (ESI) for $C_{18}H_{24}N_5O_{11}F$ Calcd (MH^+): 506.1529. Found 506.1529; Calcd (MNa^+): 528.1349. Found 528.1331.

5.1.6. 5-Fluorouridine-linker1-aminoPVA (6). A solution of **5** (18.0 mg, 0.0375 mmol), HOBt (4.8 mg, 0.0375 mmol), and EDC (13.7 mg, 0.0714 mmol) in DMF (714 μL, final concentration 0.05 M) was stirred for 60 min. Then a solution of aminoPVA in DMF/H_2O (1:1, 2 mL, 10 mg/mL, 2.5% N, 0.0375 mmol) was added and the pH adjusted to ~6.5 with 0.1 N HCl and/or 0.1 N NaOH as needed. The mixture was stirred at room temperature for 1 day. After 16 h the pH was adjusted to 6.5 if it has changed. The solution was then dialyzed (Spectra/Por® 6 dialysis membrane, MWCO 3500) against 0.2 N NaCl solution for 3 days and then against H_2O for 2 days changing the solution twice daily. Product was obtained following lyophilization as a white solid (21.4 mg).

5.1.7. Benzylamino-acetic acid 2-trimethylsilanyl-ethyl ester (10).[33,34] To a solution of Cbz-GlyCO_2H (1.046 g, 5.00 mmol) in CH_2Cl_2 (25 mL) were added DMAP (0.428 g, 3.50 mmol), 2-(trimethylsilyl)ethanol (1.07 mL, 7.50 mmol), and EDC (1.917 g, 10.00 mmol). The reaction mixture was stirred at room temperature for 1 day, then diluted with H_2O and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (8:2 hexane/EtOAc) as a clear oil (1.53 g, 99%); ^1H NMR (300 MHz, $CDCl_3$) δ 7.38–7.32 (5H, m), 5.35 (1H, br s), 5.13 (2H, s), 4.28–4.22 (2H, m), 3.96 (2H, d, J = 5.5 Hz), 1.04–0.98 (2H, m), 0.05 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 156.3, 136.3, 128.6, 128.3, 128.2, 67.1, 64.0, 43.0, 17.4, −1.4; IR (neat/NaCl) 3354, 2954, 1727, 1524, 1250, 1194 cm^{-1}; LRMS (ESI, m/z, MNa^+) 332.

5.1.8. (2-*tert*-Butoxycarbonylamino-4-methyl-pentanoylamino)-acetic acid 2-trimethylsilanyl-ethyl ester (11). To a solution of **10** (1.476 g, 4.77 mmol) in anhydrous EtOH (60 mL) was added 10% Pd/C (0.150 g). The reaction mixture was stirred under 40 psi of H_2 at room temperature for 16 h. The mixture was filtered and concentrated to give crude amine which was used in the next step without further purification (0.863 g, quant). BocLeu$CO_2H{\cdot}H_2O$ (2.033 g, 8.16 mmol) was dissolved in DMF (58 mL), to it were added HOBt (1.323 g, 9.79 mmol) and i-Pr_2NEt (2.13 mL, 12.23 mmol) and the reaction mixture was cooled in an ice bath. EDC (1.876 g, 9.79 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. To this were added the prepared crude amine (0.715 g, 4.08 mmol) and i-Pr_2EtN (2.13 mL, 12.234 mmol). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature overnight. Solvent was removed in vacuo, the residue dissolved in saturated $NaHCO_3$ and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (3:7 EtOAc/hexane) as a clear oil (0.950 g, 60%); $[\alpha]_D$ −21.8 (c 1, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 6.76 (1H, br s), 5.00 (1H, d, J = 8.0 Hz), 4.26–4.14 (3H, m), 4.00–3.97 (2H, m), 1.73–1.36 (11H, m), 1.30–1.10 (1H, m), 1.02–0.91 (8H, m), 0.03 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 169.9, 155.8, 80.2, 64.0, 53.0, 41.5, 28.4, 24.8, 23.1, 22.0, 17.4, −1.4; IR (neat/NaCl) 3307, 2956, 1751, 1665, 1528, 1173 cm^{-1}; LRMS (ESI, m/z, MH^+) 389, (MNH^{4+}) 406.

5.1.9. [2-(2-*tert*-Butoxycarbonylamino-3-phenyl-propionylamino)-4-methyl-pentanoylamino]-acetic acid 2-trimethylsilanyl-ethyl ester (12). Compound **11** (0.720 g, 1.85 mmol) was dissolved in 4 N HCl/dioxane (7 mL) and stirred at room temperature for 1 h. Solvent was removed in vacuo to give the crude amine as an HCl salt, which was used directly in the next step (0.602 g, quant). The residue (0.564 g, 1.74 mmol) was dissolved in DMF (25 mL) and to it were added HOBt (0.469 g, 3.47 mmol), *N*-methylmorpholine (1.14 mL, 10.41 mmol), and BocPheCO_2H (0.783 g, 2.95 mmol). The reaction mixture was cooled in an ice bath and to it was added EDC (0.665 g, 3.47 mmol). The mixture was allowed to warm to room temperature and was stirred for 1 day. Solvent was removed in vacuo, the crude dissolved in saturated $NaHCO_3$ and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (35:65 EtOAc/hexane) as a foamy solid (0.850 g, 91%); mp 53–56 °C; $[\alpha]_D$ −27.3 (c 1, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.26–7.13 (5H, m), 6.98 (1H, br s), 6.94 (1H, br d),

5.39 (1H, d, J = 7.4 Hz), 4.59–4.51 (1H, m), 4.47–4.40 (1H, m), 4.22–4.16 (2H, m), 3.90–3.87 (2H, m), 3.10–2.95 (2H, m), 1.73–1.42 (2H, m), 1.36 (9H, s), 1.30–1.10 (1H, m), 1.00–0.95 (2H, m), 0.87 (6H, d, J = 5.0 Hz), 0.02 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1, 171.7, 169.7, 155.7, 136.7, 129.4, 128.6, 126.8, 80.1, 63.7, 55.8, 51.6, 41.4, 40.9, 38.1, 28.3, 24.6, 22.9, 22.0, 17.3, −1.5; IR (neat/NaCl) 3291, 2956, 1752, 1690, 1649, 1527, 1174 cm^{-1}; LRMS (ESI, m/z, MH^+) 536, (MNH^{4+}) 553.

5.1.10. {2-[2-(2-*tert*-Butoxycarbonylamino-acetylamino)-3-phenyl-propionylamino]-4-methyl-pentanoylamino}-acetic acid 2-trimethylsilanyl-ethyl ester (13). Compound **12** (0.810 g, 1.51 mmol) was dissolved in 4 N HCl/dioxane (5.5 mL) and stirred at room temperature for 1 h. Solvent was removed in vacuo to give the crude amine as an HCl salt, which was used directly in the next step (0.714 g, quant). The residue (0.714 g, 1.33 mmol) was dissolved in DMF (19 mL) and to it were added HOBt (0.360 g, 2.66 mmol), *N*-methylmorpholine (0.88 mL, 7.10 mmol), and $BocGlyCO_2H$ (0.397 g, 2.26 mmol). The reaction mixture was cooled in an ice bath and to it was added EDC (0.551 g, 2.66 mmol). The reaction mixture was allowed to warm to room temperature and was stirred for 1 day. Solvent was removed in vacuo, the crude dissolved in saturated $NaHCO_3$ and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (6:4 EtOAc/hexane) as a foamy solid (0.750 g, 95%); mp 64–66 °C; $[\alpha]_D$ −25.8 (c 1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (1H, br d), 7.88 (1H, br t), 7.61 (1H, br d), 7.20–7.07 (5H, m), 6.03 (1H, br t), 5.03–4.96 (1H, m), 4.73–4.65 (1H, m), 4.23–4.18 (2H, m), 4.10–3.84 (4H, m), 3.04–2.86 (2H, m), 1.73–1.55 (2H, m), 1.41 (9H, s), 1.25–1.12 (1H, m), 1.02–0.96 (2H, m), 0.90–0.85 (6H, m), 0.03 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.2, 170.7, 169.8, 169.3, 156.3, 136.4, 129.6, 128.5, 126.8, 79.7, 63.6, 54.3, 51.6, 44.0, 41.9, 41.3, 39.4, 28.4, 24.7, 22.7, 22.6, 17.4, −1.5; IR (neat/NaCl) 3289, 2956, 1644, 1546, 1250, 1175 cm^{-1}; LRMS (ESI, m/z, MH^+) 593, (MNH^{4+}) 610.

5.1.11. 4-Azido-4-{[(1-{3-methyl-1-[(2-trimethylsilanyl-ethoxycarbonylmethyl)-carbamoyl]-butylcarbamoyl}-2-phenyl-ethylcarbamoyl)-methyl]-carbamoyl}-butyric acid 9*H*-fluoren-9-ylmethyl ester (14). Compound **13** (0.220 g, 0.37 mmol) was dissolved in 4 N HCl/dioxane (2.0 mL) and stirred at room temperature for 1 h. Solvent was removed in vacuo to give the crude amine as an HCl salt, which was used directly in the next step (0.196 g, quant). The residue (0.196 g, 0.37 mmol) was dissolved in DMF (6 mL) and to it were added HOBt (0.100 g, 0.742 mmol), *N*-methylmorpholine (0.07 mL, 0.631 mmol), and 2-azido-pentanedioic acid 5-(9*H*-fluoren-9-ylmethyl) ester[29] (0.169 g, 0.48 mmol). The reaction mixture was cooled in an ice bath and to it was added EDC (0.142 g, 0.74 mmol). The reaction mixture was allowed to warm to room temperature and was stirred for 1 day. Solvent was removed in vacuo, the crude dissolved in saturated $NaHCO_3$ and extracted with CH_2Cl_2 (4×). The combined organic layers were dried ($MgSO_4$) and concentrated. Product was obtained following column chromatography (8:2 EtOAc/hexane) as a foamy solid (0.200 g, 65%); mp 140 °C, dec.; $[\alpha]_D$ −18.8 (c 1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (3H, br s), 8.42 (1H, br s), 7.77 (2H, d, J = 7.4 Hz), 7.68–7.63 (2H, m), 7.42–7.30 (4H, m), 7.21–7.10 (5H, br s), 5.26 (1H, br s), 4.91 (1H, br s), 4.52–4.38 (3H, m), 4.32–3.92 (7H, m), 3.10 (1H, br s), 2.92 (1H, br s), 2.66 (2H, br s), 2.28 (2H, br s), 1.67 (2H, br s), 1.41 (1H, br s), 1.02–0.84 (8H, m), 0.02 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 172.4, 170.7, 170.0, 169.3, 168.2, 143.7 (2), 141.3, 136.2, 129.8, 128.5, 127.9, 127.2, 126.8, 125.1, 120.2, 66.8, 63.8, 60.6, 54.4, 51.5, 46.8, 43.0, 41.6, 40.7, 30.6, 27.2, 24.6, 23.4, 22.0, 17.4, −1.5; IR (neat/NaCl) 3278, 2956, 2103, 1739, 1634, 1543 cm^{-1}; LRMS (ESI, m/z, MH^+) 826.

5.1.12. 4-Azido-4-(GFLG-DOX)-butyric acid (15). Compound **14** (0045 g, 0.054 mmol) was dissolved in TFA/CH_2Cl_2 1:1 (1.0 mL) and stirred at room temperature for 80 min. Solvent was evaporated and then traces of TFA were removed by co-evaporating with MeOH. The carboxylic acid was used in the next step without further purification (0.039 mg, quant). The carboxylic acid (0.039 g, 0.054 mmol) was dissolved in DMF (6.0 mL) and to it were added HOBt (0.018 g, 0.13 mmol) and EDC (0.031 g, 0.16 mmol). The reaction mixture was stirred at room temperature for 30 min and then doxorubicin·HCl (0.031 g, 0.054 mmol) and NEt_3 (0.03 mL, 0.22 mmol) were added and the reaction mixture was stirred in the dark for 1 day. The crude reaction was diluted with H_2O and extracted with CH_2Cl_2 (4×). The organic layers were washed with brine, dried ($MgSO_4$), and concentrated. The product was obtained following column chromatography (5:95 MeOH/EtOAc) as a red solid (0.045 g, 67%); $[\alpha]_D$ +76.0 (c 0.312, CH_3OH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36–8.33 (1H, m), 8.14 (1H, t, J = 7.0 Hz), 7.94 (1H, t, J = 6.0 Hz), 7.87–7.82 (4H, m), 7.63 (2H, d, J = 7.4 Hz), 7.60–7.56 (1H, m), 7.50 (1H, d, J = 8.2 Hz), 7.40 (2H, t, J = 7.4 Hz), 7.31 (2H, t, J = 7.4 Hz), 7.22–7.19 (5H, m), 7.16–7.12 (1H, m), 5.43 (1H, s), 5.22 (1H, s), 4.90–4.86 (2H, m), 4.80 (1H, d, J = 6.0 Hz), 4.58 (2H, d, J = 5.9 Hz), 4.56–4.50 (1H, m), 4.37 (2H, d, J = 6.8 Hz), 4.26–4.16 (3H, m), 4.00–3.93 (4H, m), 3.84 (1H, t, J = 6.9 Hz), 3.76–3.58 (4H, m), 3.41–3.38 (1H, m), 3.02–2.84 (2H, m), 2.76–2.66 (1H, m), 2.41 (1H, t, J = 7.6 Hz), 2.22–2.18 (1H, m), 2.11–2.06 (1H, m), 1.92–1.81 (2H, m), 1.56–1.50 (1H, m), 1.48–1.40 (3H, m), 1.13 (3H, d, J = 6.4 Hz), 0.84–0.77 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ 186.4, 186.3, 172.0, 171.9, 170.9, 169.2, 168.1, 167.8, 160.7, 156.1, 154.5, 143.6 (2), 140.7, 137.7, 136.2, 135.4, 134.6, 134.0, 129.2, 128.0, 127.7, 127.2, 126.2, 125.0, 120.1, 119.9, 119.6, 118.9, 110.7, 110.6, 100.4, 74.9, 70.0, 68.0, 66.6, 65.6, 63.7, 60.6, 56.5, 53.8, 51.2, 46.2, 45.1, 42.0, 41.7, 40.7, 37.5, 36.6, 32.0, 29.8, 26.6, 24.1, 22.9, 21.6, 17.0; IR (neat/NaCl) 3281, 2927, 2106, 1728, 1631, 1446, 1284 cm^{-1}; LRMS (ESI, m/z, MH^+) 1252; HRMS (ESI) for $C_{51}H_{60}N_8O_{18}$ Calcd (MNa^+): 1273.4700. Found 1273.4681. The red solid (0.023 g, 0.018 mmol) was then dissolved in DMF (0.4 mL) and

to it was added *N*-methylpyrrolidine (0.2 mL) and the reaction mixture was stirred in the dark at room temperature for 1 h. The solvent was removed in vacuo, the crude product dissolved in small amount of CH_2Cl_2 and MeOH, then Et_2O was added dropwise and with stirring to precipitate the product. The mixture was centrifuged and the supernatant was discarded. After centrifugation the pellet was isolated and dried. Product was obtained following column chromatography (7:3 EtOAc/MeOH) as a red solid (0.014 g, 70%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.52–8.30 (3H, m), 7.97–7.79 (2H, m), 7.73–7.66 (1H, m), 7.59–7.52 (1H, m), 7.41–7.35 (1H, m), 7.26–7.11 (5H, m), 5.28–5.22 (1H, m), 4.95 (1H, br s), 4.61–4.46 (2H, m), 4.28–4.11 (2H, m), 4.02–3.81 (4H, m), 3.73–3.56 (4H, m), 3.47–3.23 (4H, m), 3.04–2.79 (3H, m), 2.26–2.08 (3H, m), 2.01–1.79 (3H, m), 1.61–1.40 (4H, m), 1.25–1.03 (4H, m), 0.90–0.74 (6H, m); IR (neat/NaCl) 3334, 2953, 2107, 1651, 1574, 1410, 1286 cm^{-1}; HRMS (ESI) for $C_{51}H_{60}N_8O_{18}$ Calcd (MNa^+): 1095.3918. Found 1095.3888.

5.1.13. DOX-linker-aminoPVA (16). A solution of **15** (13.4 mg, 0.0125 mmol), HOBt (1.7 mg, 0.0125 mmol) NEt_3 (3.5 μL, 0.0250 mmol), and EDC (4.8 mg, 0.0250 mmol) in DMF (240 μL, final concentration 0.05 M) was stirred for 60 min. Then a solution of aminoPVA in DMF/H_2O (1:1, 0.7 mL, 10 mg/mL, 2.5% N, 0.0125 mmol) was added and the pH adjusted to ~8.3 with 0.1 N HCl and/or 0.1 N NaOH. The mixture was stirred at room temperature for 1 day. The solution was then dialyzed (Spectra/Por® 6 dialysis membrane, MWCO 3500) against 0.2 N NaCl solution for 3 days and then against H_2O for 2 days changing the solution twice daily. Product was obtained following lyophilization as a red solid (9.1 mg).

5.2. Preparation of drug-functionalized-SPIONs

Superparamagnetic iron oxide nanoparticles (ferrofluid) were prepared according to previously described methods.[22–24,35,36] Briefly, ferrofluid was prepared by alkaline co-precipitation of ferric (0.086 M) and ferrous (0.043 M) chlorides (Fluka, Buchs, Switzerland). After washing with water, the black precipitate was refluxed in 0.8 M nitric oxide–0.21 M $Fe(NO_3)_3{\cdot}9H_2O$ (Fluka, Buchs, Switzerland) for 1 h, cooled, and the brown precipitate was dispersed in H_2O and dialyzed for 2 days against 0.01 M HNO_3.

To obtain aminoPVA-SPIONs the ferrofluid was mixed with poly(vinylalcohol) (PVA 3-83, Mowiol, MW 7600 g/mol, courtesy of Clariant) and poly(vinylalcohol/vinylamine) (aminoPVA M12, Erkol, MW 20,000 g/mol, courtesy of Erkol) as described previously[22,24] and the pH was adjusted to 7.0. For the experiments described here, the ratio of polymer to iron was 10 and the ratio of PVA to aminoPVA copolymer was 45 (mass ratios).

For the preparation of drug-functionalized-SPIONs, the ferrofluid was mixed in ultrapure water with PVA, aminoPVA copolymer, and drug-linker-aminoPVA at various ratios (see Table S2). The drug-functionalized-SPIONs were stabilized at pH 6–7, adjusted with diluted HNO_3 or NH_3.

5.3. Characterization of drug-functionalized-SPIONs

Particle size distributions were measured by photon correlation spectroscopy (PCS) using a Brookhaven apparatus equipped with a BI-9000AT digital autocorrelator instrument and a He–Ne laser beam at a wavelength of 661 nm (scattering angle of 90°). The CONTIN method was used for data processing. Viscosity and refraction index of pure water were used for size distribution calculation. Coated-SPIONs were diluted 51-fold in 0.01 M HNO_3 in ultrapure water pre-filtered on 20 nm ceramic filters (Whatman, Anodisc 25). The same setting equipped with platinum electrodes was used for electrophoretic mobility measurements and zeta potential was calculated using the Smoluchowski approximation. The electrodes were cleaned for 5 min in an ultrasonic bath prior to each measurement.

Size distribution was also determined by gel-filtration. Samples of 15 μL of aminoPVA-SPIONs or drug-SPIONs were applied to a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column (0.6 × 19 cm) equilibrated in 25 mM $NaH_2PO_4{\cdot}2H_2O$–0.15 M NaCl, pH 7.0–7.1 (PBS), pre-filtered through a 0.22 μm filter (Millex® GP, Millipore), at a flow rate of 20 mL/h and room temperature. The fractions were analyzed for iron, polyvinylalcohol, and fluorescence. For iron determination 50 μL of each fraction was mixed with 50 μL, 6 N HCl for 1 h at room temperature, and 100 μL of a 5% solution of $K_4[Fe(CN)_6{\cdot}3H_2O]$ was added for 30 min. Absorbance was read at 690 nm in a multiwell-plate reader (Labsystems iEMS Reader MF, BioConcepts, Allschwil, Switzerland) and iron was quantified from a standard curve of aminoPVA-SPIONs treated in same conditions. For PVA quantification, to 20 μL of each fraction, 110 μL of distilled water and 70 μL of an iodine solution (0.45% KI, 0.225% I_2, and 3.6% H_3BO_3 (w/v)) were added and incubated at room temperature for 30 min. Absorbance was read at 690 nm in a multiwell-plate reader and the amount of PVA was quantified from a standard curve of PVA treated in same conditions. The fluorescence of DOX in 6 N HCl was measured at $\lambda_{ex}/\lambda_{em}$ of 485/580 nm in a multiwell-plate reader (CytoFluor, Series 4000, PerSeptive Biosystems).

5.4. Biological assays

5.4.1. Enzymatic digestion of DOX-SPIONs 16S and evaluation of drug release. For the evaluation of the release of DOX from DOX-SPIONs **16S** by cellular proteases, human melanoma cells (Me300, see below) were grown to confluence in Petri dishes and thiol-cathepsins were extracted from the cell layer in 0.1 M $Na_2HPO_4{\cdot}2H_2O$, 2 mM EDTA, 8 mM DTT, pH 6.0, at 4 °C by 3 cycles of freeze–thaw in liquid nitrogen. Cathepsin B activity in the extract was controlled with the substrate essentially as previously described.[31] Then 2 volumes of DOX-SPIONs **16S** were exposed for 24 h at 37 °C to 1 volume of cell extract, centrifuged 5 min at 1000 rpm, and 20 μL of supernatant was applied to a

Sephadex G-75 column in phosphate buffer, pH 7. The eluting fractions were monitored for iron, PVA, and DOX, as described above.

5.4.2. Cells and cell treatment. The Me300 human melanoma cell line (a kind gift of D. Rimoldi, Ludwig Institute, Lausanne, Switzerland) was grown in RPMI 1640 medium containing 10% FCS and antibiotics (all from Gibco, Invitrogen, Basel, Switzerland) at 37 °C and 6% CO_2. Two to three days prior to experiments, the cells were detached with trypsin–EDTA (Gibco), centrifuged, and grown in complete medium in multiwell-plates (Costar, Corning, NY, USA), in order to reach 60–80% confluence on the day of experiment. Fresh complete medium was added prior to exposure to the drugs for the concentration and time indicated. Fur was obtained from Sigma (Steinheim, Germany) and 25 mM stock solutions were prepared in PBS. DOX solution for clinical use (Adriblastin solution, 10 mg/5 mL in 0.9% NaCl) was purchased from Pfizer AG. Experiments were performed in triplicate wells. Thymidine incorporation to quantify DNA synthesis and Alamar Blue reduction to determine cell viability were performed (see below). Alternatively, the cell layers were washed twice with saline and cellular iron content was quantified using the Prussian Blue method and drug content by measuring drug fluorescence (see below). Cell number was determined by counting in a hemocytometer under a phase-contrast microscope and protein content was determined in cell extracts using the BCA reagent (Pierce, Socochim, Lausanne, Switzerland).

5.4.3. Evaluation of cell viability and DNA synthesis. To quantify metabolically active cells, the cells were exposed to 10% Alamar Blue (Serotec, Düsseldorf, Germany) added to the cell culture medium and fluorescence increase was directly measured in a multiwell fluorescence reader ($\lambda_{ex}/\lambda_{em}$ = 530/580 nm) after 2 h at 37 °C. To assess DNA synthesis 1 μCi/mL [^{3}H]thymidine (Amersham Pharmacia, Dübendorf, Switzerland) was added for the last 2 h of exposure to compounds and incorporation was quantified in a beta-counter (Rackbeta, LKB) after precipitation with 10% trichloroacetic acid and solubilization in 0.1 N NaOH–1% SDS.

5.4.4. Total cell-bound iron and drug determination. To quantify cellular iron content, the cell layer was dissolved for 1 h in 6 N HCl (125 μL/well of a 48-well plate), then 125 μL of a 5% solution of $K_4[Fe(CN)_6]\cdot 3H_2O$ in H_2O was added for 20 min and the absorbance was read at 690 nm in a multiwell-plate reader. A standard curve of aminoPVA-SPIONs in 6 N HCl was treated in the same conditions to quantify the amount of cell-bound iron. Cellular drug uptake was determined in lysed cells by measuring DOX fluorescence intensity in 6 N HCl at $\lambda_{ex}/\lambda_{em}$ of 485/580 nm in a multiwell fluorescence plate reader. To calculate the number of SPIONs added to cells or taken up by cells, a mean diameter of 9 ± 3 nm, corresponding to a mean iron content of 1.3×10^{-15} mg Fe per particle, was calculated according to:[22]

$$N_{6\text{–}12} = 100 \cdot \frac{M_{Fe}}{M_T(Fe)}$$

M_T(Fe): total iron (Fe) mass of all 100 nanoparticles sample [mg];

M_{Fe}: mass of iron (Fe) in a sample (measured by Prussian Blue reaction) [mg];

$N_{6–12}$: amount of Fe_2O_3 nanoparticles in M_{Fe} mg of iron (Fe) in a sample, calculated between 6 and 12 nm range.

5.4.5. Calculation of results. Each experiment was repeated in triplicate wells at least twice. Means and standard deviation were calculated.

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Supplementary Material

The complete syntheses of compounds **7**, **8**, and **9**, as well as Schemes S1, S2, and S3 and Tables S1 and S2 are provided as supplementary material. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.12.051.

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