Bioorganic & Medicinal Chemistry Letters 20 (2010) 5454–5458

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

$^{72/74}$As-labeling of HPMA based polymers for long-term in vivo PET imaging

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ARTICLE INFO

Article history:
Received 29 May 2010
Revised 20 July 2010
Accepted 21 July 2010
Available online 25 July 2010

Keywords:
$^{72/74}$As-labeling
Long-term imaging
PET
Polymers

ABSTRACT

In the context of molecular imaging, various polymers based on the clinically approved *N*-(2-hydroxypropyl)-methacrylamide (HPMA) have been radio-labeled using longer-living positron emitters ^{72}As $t_{1/2}$ = 26 h or ^{74}As $t_{1/2}$ = 17.8 d. This approach may lead to non-invasive determination of the long-term in vivo fate of polymers by PET (positron emission tomography). Presumably, the radio label itself will not strongly influence the polymer structure due to the fact that the used nuclide binds to already existing thiol moieties within the polymer structure. Thus, the use of additional charges or bulky groups can be avoided.

The field of polymer-based therapeutics has seen a tremendous increase in interest during the last decades.[1–7] Under the term nanomedicine, various systems have entered a broad range of clinical research. One reason for this is the promising opportunity to combine various functionalities among one particle. Although the idea of polymer-based nanomedicine sound promising, further research with a view towards clinical application is essential to gain detailed knowledge about short- as well as long-term biodistribution of particles. A precise biodistribution is essential to understand in vivo fate. This knowledge is of major importance when particle properties need to be subsequently enhanced. To date, it is already well known that particle properties such as size, charge, and surface characteristics determine the in vivo fate as well as cellular uptake and intracellular distribution.[8,9] Thus, these properties of nanoparticles offer the possibility to tune the body distribution, for example, in cancer therapy via the enhanced permeability and retention effect (EPR-effect; passive targeting)[6,10] or by attaching various selective targeting vectors (active targeting).[4] In addition, encapsulation of drugs, proteins or oligonuclides within these polymeric structures and controlled release afterwards bear the possibility to reduce the toxicity of various therapies due to reduced unspecific release. However the concept itself already led to polymer therapeutics in clinical trails.[11–13]

The first polymer drug conjugate approved by the FDA (USA—Food and Drug Administration) for clinical trails was based on *N*-(2-hydroxypropyl)-methacrylamide (HPMA).[3,11,12]

Until now, most HPMA polymer drug conjugates have been synthesized using free radical polymerization techniques, that suffer from a lack of end group control and have broad molecular weight distributions. Fractionation techniques can improve the broad distribution but fail in controlling polymer end groups as well as separating macromolecules bearing different end groups. In addition, a loss of product has to be accepted. Controlled radical polymerization (CRP) techniques are currently able to tackle all these points. Furthermore, they open the road to more complex polymer architectures, for example, block copolymers or star-shaped polymers.[14–16]

Amphiphilic block copolymers are particularly interesting due to their aggregation into defined superstructures in aqueous solution, which can be used to encapsulate hydrophobic substances. These substances can then be delivered to the site of action whilst avoiding metabolism.[13–17] In addition, Barz et al. have recently reported that the cellular uptake kinetics in human multi-drug resistant breast adenocarcinoma cells can be strongly influenced by variations in the polymer architecture.[9] Thus, structural variations can be used to increase or reduce unspecific cellular uptake.

To understand and fine-tune structural parameters as well as active targeting in vivo appropriate imaging strategies are needed. Positron emission tomography (PET) is a non-invasive, quantitative, and repetitive whole body molecular imaging technique. PET can thus be considered as a promising approach to understand the mentioned interactions. The most frequently used radioactive nuclides for in vivo imaging of nanoparticles are chelated metals, such as ^{64}Cu.[18,19] This strategy may have a major drawback, where the chelating agent itself is large, bulky, and charged and therefore may change the properties of the polymeric particle itself. As a result, the fate of polymer when used in vivo will be strongly

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doi:10.1016/j.bmcl.2010.07.092

influenced by the radioactive label. Consequently, its biological behavior may differ from the one of the original nanoparticle. Ideally, the radioactive label should be as 'inert' as possible. In this context, Herth et al.[20] as well as Weissleder and co-workers[21] recently described a new ^{18}F-labeling strategy via the introduction of [^{18}F] radionuclides to image the short-term in vivo distribution of polymeric particles. However, [^{18}F] has a relatively short $t_{1/2}$ of ~110 min and thus it may be useful in the development of polymeric imaging agents but has limited use when studying long-term polymer distribution or accumulation in a certain tissue (EPR-effect). For those studies a time frame of weeks to month is desirable and therefore additional long-term imaging is necessary.

^{72}As is a pure β^+-emitter with a $t_{1/2}$ = 26 h, whereas ^{74}As decays via β^+-emission (29%) and electron capture (66%) with a $t_{1/2}$ = 17.8 d. Both radioisotopes provide physical half-lives to follow biological processes for several days (^{72}As) or approximately two months (^{74}As).

Arsenic and many of its compounds are especially potent poisons. The acute minimal lethal dose of arsenic in adults is estimated to be 70–200 mg or 1 mg/kg/day.[22] However, the amount of 100 MBq ^{72}As or ^{74}As administered during a PET-Scan is estimated to be ~74 ng (1 nmol)–0.007 ng (1 pmol) and therefore, toxic effects are not expected.

Herein, we report the $^{72/74}$As-labeling of HPMA based polymeric structures with a view towards non-invasively long-term imaging of polymeric particles in vivo offering great potential to study polymer related effects such as the passive polymer accumulation in tumor tissue (EPR-effect) or active targeting.

As(III) is known to bind to thiol groups covalently. Therefore, thiols display an adequate precursor moiety for labeling with $^{72/74}$As.[23] Due to the mechanism of the reversible addition fragmentation chain transfer (RAFT) polymerization and the use of dithiobenzoic ester as a chain tranfer agent each polymeric chain bears a dithiobenzoate (Scheme 1). These endgroups can be converted afterwards into thiols by either an aminolysis or reduction. However, this reaction leads to the formation of by-products, for example, disulfides or thiolactones, which have to be reduced to derive free thiols, cf. Scheme 1.

Radiolabeled [^{72,74}As] monoclonal antibodies have recently been used to evaluate the molecular targeting mechanism of anti-phosphatidyl-directed tumor therapeutics.[24] In summary, ^{72}As and ^{74}As have advantages due their half-lives and their binding mode.

The synthesis of well defined poly(HPMA) homopolymers **P1** and **P2**, poly(HPMA)-block-poly(lauryl methacrylate) block copolymers **P3** and poly(HPMA)-random-poly(ethylmethyl disulfide acrylamide) **P4** copolymers was performed according to the recently published method of Barz et al. (Table 1, Scheme 1).[25–32]

In addition, another polymer (**P4**) was synthesized containing a higher number of disulfide side chains, which can be reduced by (tris(2-carboxyethyl)phosphine) (TCEP) resulting in thiol units (Scheme 2). This results in a higher number of precursor groups that are accessible and in principle should lead to higher corrected radioactive labeling yields (RCY's).

As mentioned above disulfides can be formed during the aminolysis of the dithiobenzoic ester, which has to be reduced in order to enhance the radiochemical yield. Therefore, all polymers were reduced using TCEP thus forming polymers bearing free thiol groups, which could be purified from side products of the reduction by size exclusive chromatography (SEC) (HiTrap Desalting Column, Sephadex G-25 Superfine, column volume 5 mL; flow rate: 0.5 mL 0.9% NaCl-solution) and used in the next step for radioactive labeling (Scheme 3).

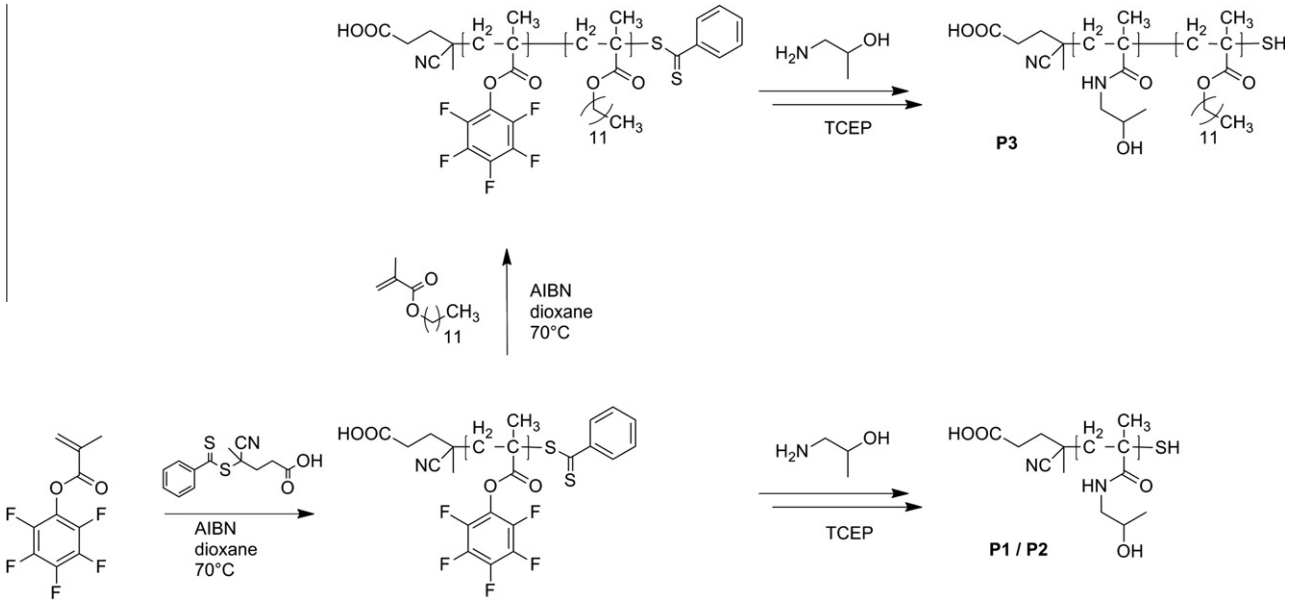
$^{72/74}$As were produced by (p,n) bombardment of ^{nat}Ge at the (DKFZ) cyclotron in Heidelberg. The protons used had an energy of 15 MeV and a beam current of up to 30 μA. Yields of 4 GBq of ^{72}As and 400 MBq of ^{74}As were obtained with 200 μAh.

The radio-arsenic was separated from bulk amounts of the germanium target material in a multi step separation procedure.[33] In the first step the most of the germanium was separated from the

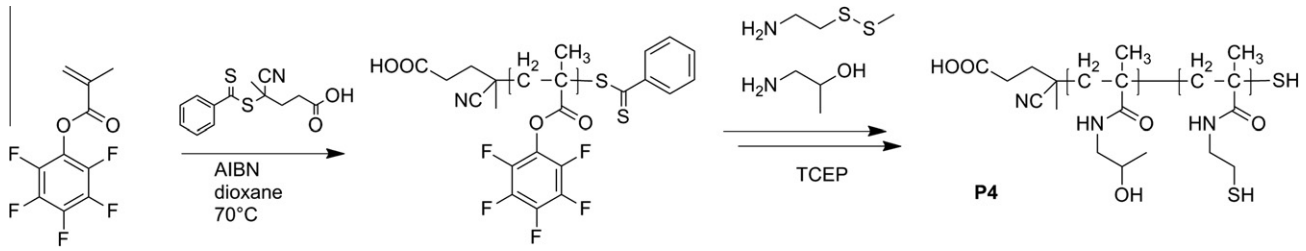
Table 1
Characteristics of synthesized polymers for labeling with $^{72/74}$As

Polymer	Structure	Thiol content[a] (%)	M_n[b]	M_w[b]	PDI[b]
P1	Homo polymer	1.1	12.5	15.7	1.26
P2	Homo polymer	0.50	27.1	33.6	1.24
P3	Block copolymer	0.48	27.7	32.5	1.26
P4	Random copolymer	9.6	27.0	33.5	1.24

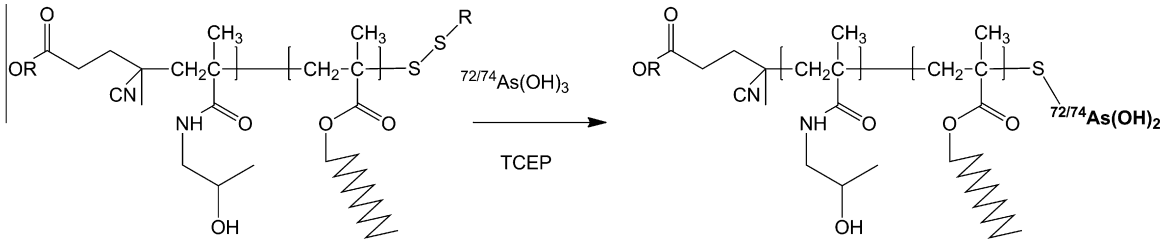
[a] Calculated from the degree of polymerization and ^{1}H NMR.
[b] kg/mol, calculated from the molecular weight of the reactive precursors, which was determined by GPC in THF as the solvent.



Scheme 1. Synthesis of HPMA based polymers bearing thiol end groups for radioactive labeling with $^{72/74}$As.



Scheme 2. Synthesis of HPMA based polymers **P4** with an increased content of free thiol groups along the polymer backbone for radioactive labeling with $^{72/74}$As.



Scheme 3. $^{72/74}$As-Labeling of HPMA based polymers.

radio-arsenic by distillation of $GeCl_4$ from 10 M HCl at 120 °C as the $^{72/74}$As is not volatile in the oxidation state (V) and stays inside the distillation vessel. In the second step, the $^{72/74}$As(V) was purified from remaining traces of germanium by anion exchange chromatography yielding the arsenic in about 500 µL 10 M HCl. Reduction to $^{72/74}$As(III) was carried out by boiling the solution at 60 °C for 1 h after the addition of 10 mg CuCl. The $^{72/74}$As(III) was extracted into 500 µL CCl_4 followed by back extraction into 500 µL of PBS-buffer at pH 7. This solution was directly used for labeling experiments.

$^{72/74}$As-labeling of HPMA based polmers (**P1**, **P2**, and **P3**) was carried out in H_2O at 70 °C. Therein, 0.1 mmol (3 mg) of polymer **P2** was dissolved in 0.5 mL H_2O/DMSO and 10 µmol (3 mg) of TCEP was added to reduce disulfide bonds into the desired thiols. This mixture was stirred for at least 1 h before the radioactive nuclides dissolved in 0.5 mL PBS-buffer were added to the reduced polymers. Radiochemical yields were analyzed at different time points via SEC. Figure 1 shows an representative elution profile of the SEC.

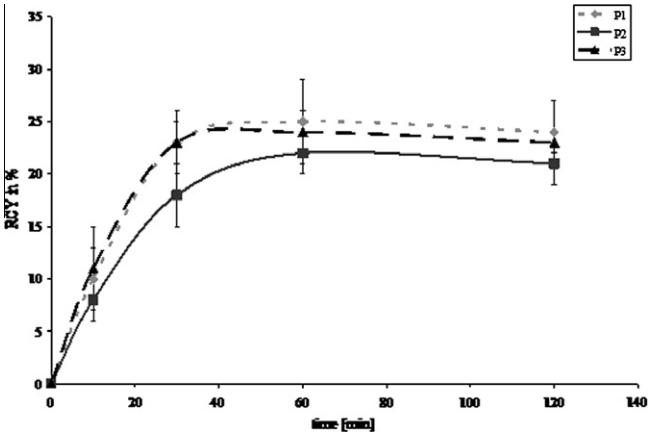


Figure 2. Time dependency of the radio-arsenic labeling yields of **P1**, **P2**, and **P3** ($n = 3$).

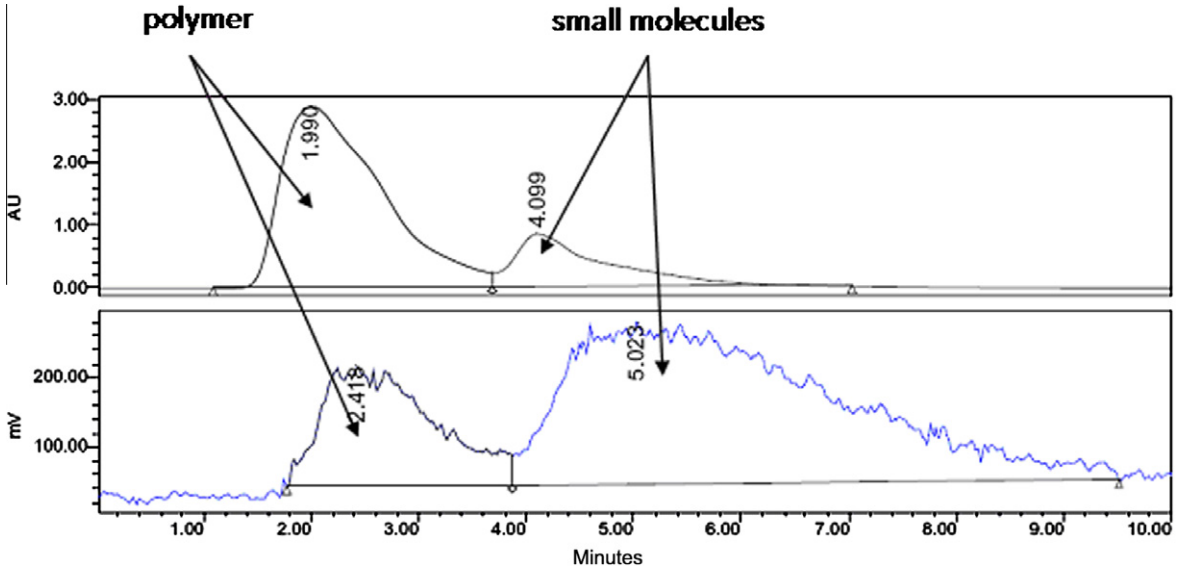


Figure 1. SEC-elution profile of **P1**; channel 1: UV absorption, channel 2: radioactivity detection.

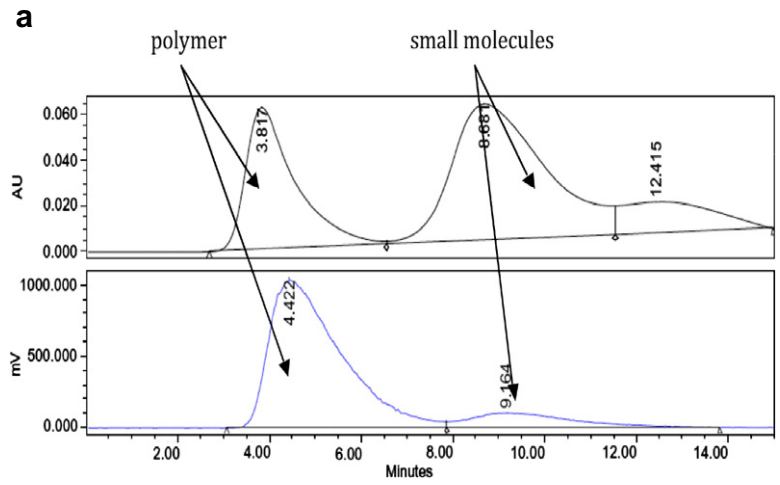


Figure 3. ^{72}As-Labeling of 0.1 mmol **P4** at 30 °C using H_2O as a solvent ($n = 4$). (a) SEC-elution profile of **P4**; channel 1: UV absorption, channel 2: radioactivity detection. (b) Time dependency of the RCY.

RCY's of ~20% could be obtained, whereas Figure 2 shows the time dependency of the reaction.

Thereby, specific activities (A_s) of ~10^{-4} GBq/μmol with a batch activity of 100 MBq were obtained.

The radioactive labeling of polymer end groups has some advantages. First of all each polymer has only one reactive side, reducing the possibility of undesired side reactions such as the crosslinking of two polymers. In addition, the influence on polymer aggregation in solution related to the attachment of the arsenic compound is reduced dramatically. In the performed experiments no crosslinking could be detected by GPC and additionally, no chance in the polymer properties was observed. These findings appear reasonable because only a certain number of polymer end-groups were labeled with the arsenic radionuclide.

In addition, we also synthesized a polymer (**P4**) bearing a higher number of disulfide side chains, which can be reduced by TCEP resulting in thiol units (Scheme 2). Higher numbers of precursor groups were thus accessible and should in principle lead to higher RCY's (Fig. 3). However, this approach will ultimately influence the polymer structure and therefore the aggregate in solution. Nevertheless, these studies will give an insight into the influence of thiol content on the RCY.

In the case of **P4** the labeling procedure was slightly modified. Firstly, 3 mg (0.1 mmol) of **P4** was dissolved in 500 μL 0.9% NaCl-solution and incubated for at least 1 h with 3 mg (11 mmol) TCEP at room temperature. During this time the disulfide bonds were cleaved and mercaptomethane was formed. As the arsenic As(III) reacts with the thiol groups. The mercaptomethane was removed before the labeling procedure by SEC (HiTrap Desalting Column, Sephadex G-25 Superfine, column volume 5 mL; flow rate: 0.5 mL 0.9% NaCl-solution). The purified **P4** was obtained in 1 mL of 0.9% NaCl-solution and added directly to the $^{72/74}$As(III) in 500 μL PBS-buffer. To prevent the thiols from reoxidizing, 10 μL TCEP (410 nmol) was added to the solution. The incubation was carried out at 30 °C and the yield was monitored at various time points via SEC. Figure 3 demonstrates the high RCY of ~90% in 150 min. Thereby, specific activities (A_s) of ~10^{-3} GBq/μmol with a batch activity of 100 MBq were obtained.

The stability of the labeled polymers was investigated in isotonic saline at room temperature. For this purpose the water stored polymers were reinjected into a SEC column (HiTrap Desalting Column, Sephadex G-25 Superfine, column volume 5 mL; flow rate: 0.5 mL 0.9% NaCl-solution) and checked for impurities. Even 48 h after the initial purification, no decomposition was observed.

In conclusion, $^{72/74}$As-labeling of HPMA based polymers is a new approach for labeling polymer structures. The technique was carried out using trivalent As(III) and utilizing free thiols on the polymer leading to high RCYs. In combination with a stability of at least 48 h in saline, the labeled conjugates will lead to the possibility to perform in vivo long-term μPET studies with a minimized influence on the polymer structure. Therefore, this approach may contribute to the understanding of how the alterations in physical properties of the nanostructures such as size, surface chemistry or core material influence the fate of nanoparticles in vivo. Detailed studies regarding the in vivo stability as well as initial in vivo experiments are warranted and currently under investigation.

Acknowledgments

The authors wish to thank H. Hauser and Professor Dr. M. Eisenhut for the production of $^{72/74}$As at the DKFZ in Heidelberg. We also want to thank Professor Dr. H. Ringsdorf and Dr. Richard England for their advice and fruitful discussion. Financial support by Friedrich-Naumann-Stiftung, the European Network of Excellence (EMIL), and Polymat Graduate School of Excellence is gratefully acknowledged.

References and notes

1. Ferrari, M. *Nat. Rev. Cancer* **2005**, *5*, 161.
2. Ringsdorf, H. *J. Polym. Sci. Polym. Symp.* **1975**, *51*, 135.
3. Duncan, R. *Nat. Rev. Cancer* **2006**, *6*, 688.
4. Haag, R.; Kratz, F. *Angew. Chem., Int. Ed.* **2006**, *45*, 1198.
5. Duncan, R. *Pharm. Sci. Technol. Today* **1999**, *2*, 441.
6. Matsumura, Y.; Maeda, H. *Cancer. Res.* **1986**, *46*, 6387.
7. Duncan, R. *Nat. Rev. Drug Disc.* **2003**, *2*, 347.
8. Gratton, S.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 11613.
9. Barz, M.; Luxenhofer, R.; Zentel, R.; Kabanov, A. V. *Biomaterials* **2009**, *30*, 5682.
10. Maeda, H.; Wu, J.; Sawa, Y.; Matsumura, Y.; Hori, K. *J. Controlled Release* **2000**, *65*, 271.
11. Vasey, P. A.; Kaye, S. B.; Morrison, R.; Twelves, C.; Wilson, P.; Duncan, R.; Thomson, A. H.; Murray, L. S.; Hilditch, T. E.; Murray, T.; Burtles, S.; Fraier, D.; Frigerio, E.; Cassidy, J. *Clin. Cancer Res.* **1999**, *5*, 83.
12. Hopewell, J. W.; Duncan, R.; Wilding, D.; Chakrabarti, K. *Hum. Exp. Toxicol.* **2001**, *20*, 461.
13. Matsumura, Y.; Kataoka, K. *Cancer Sci.* **2009**, *100*, 572.
14. Tsarevsky, N. V.; Matyjaszewski, K. *Chem. Rev.* **2007**, *107*, 2270.
15. Moad, G.; Rizzardo, E.; Thang, S. H. *Polymer* **2008**, *49*, 1079.
16. Boyer, C.; Bulmus, V.; Davis, T. P.; Ladmiral, V.; Liu, J.; Perrier, S. *Chem. Rev.* **2009**, *109*, 5402.

17. Kabanov, A. V.; Batrokova, E. V.; Alakhov, V. Y. *J. Controlled Release* **2002**, *82*, 189.
18. Rossin, R.; Muro, S.; Welch, M. J.; Muzykantov, V. R.; Schuster, D. P. *J. Nucl. Med.* **2008**, *49*, 103.
19. Nahrendorf, M.; Zhang, H.; Hembrador, S.; Panizzi, P.; Sosnovik, D. E.; Aikawa, E.; Libby, P.; Swirski, F. K.; Weissleder, R. *Circulation* **2008**, *117*, 379.
20. Herth, M. M.; Barz, M.; Moderegger, D.; Allmeroth, M.; Jahn, M.; Thews, O.; Zentel, R.; Rösch, F. *Biomacromolecules* **2009**, *10*, 1697.
21. Devaraj, N. K.; Keliher, E. J.; Thurber, G. M.; Nahrendorf, M.; Weissleder, R. *Bioconjugate Chem.* **2009**, *20*, 397.
22. Dart, R. C. *Medical Toxicology*; Medical Toxicology: Philadelphia, 2004. pp 1393–1401. ISBN 0-7817-2845-2.
23. Jennewein, M.; Hermanne, A.; Mason, R. P.; Thorpe, P. E.; Rösch, F. *Nucl. Instrum. Methods Phys. Res., Sect. A* **2006**, *569*, 512.
24. Jennewein, M.; Lewis, M. A.; Zhao, D.; Tsyganov, E.; Slavine, N.; He, J.; Watkins, L.; Kodibagkar, V. D.; O'Kelly, S.; Kulkarni, P.; Antich, P. P.; Hermanne, A.; Rösch, F.; Mason, R. P.; Thorpe, P. E. *Clin. Cancer Res.* **2008**, *14*, 1377.
25. Barz, M.; Tarantola, M.; Fischer, K.; Schmidt, M.; Luxenhofer, R.; Janshoff, A.; Zentel, R. *Biomacromolecules* **2008**, *9*, 3114.
26. Barz, M.; Canal, F.; Koynov, K.; Zentel, R.; Vicent, M. J. *Biomacromolecules*, doi:10.1021/bm100338x.
27. The homo and blockcopolymers were synthesized according to 9.
28. Synthesis of homopolymer precursors (**1**). The RAFT polymerizations of the PFMA using 4-cyano-4-((thiobenzoyl) sulfanyl) pentanoic acid were performed in a schlenk tube. The reaction vessel was loaded with 2,2′-azobis(isobutyronitrile (AIBN), 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid (CTA) (molar ratio of AIBN/CTA = 1:8) and 15 g of PFMA in 20 mL of dioxane. Following three freeze–vacuum–thaw cycles, the tube was immersed in an oil bath at 70 °C. Afterwards the polymer poly(PFMA) was precipitated three times into hexane, then isolated by centrifugation and dried for 12 h at 30 °C under vacuum. A slightly red powder was obtained. Yield: (59%). ^{1}H NMR ($CDCl_3$): 1.6–2.2 (br), 0.9–1.5 (br) δ (ppm) ^{19}F NMR ($CDCl_3$): δ (ppm) −165.0 (br), −159.7 (br), −154.4 (br), −153.1 (br).
29. General synthesis of block copolymer precursors (**2**). The macro CTA obtained in the above-mentioned polymerization was dissolved in dioxane and then AIBN was added. Nitrogen was bubbled through the solution and three freeze–vacuum–thaw cycles were applied. Following this, the tube was immersed in an oil bath at 70 °C. After a polymerization time of 12 h, the solution was slightly concentrated and precipitated twice in ethanol and diethyl ether, removed by centrifugation and dried overnight at 30 °C in vacuo. A slightly red powder was obtained. Yield: (89%). ^{1}H NMR ($CDCl_3$): δ (ppm) 1.6–2.2 (br), 0.9–1.5 (br), 0.8–0.9 (br t) ^{19}F NMR ($CDCl_3$): δ (ppm) −165.2 (br), −159.8 (br), −154.4 (br), −153.1 (br).
30. Postpolymerization modification of homo polymers (**3**). In a typical reaction 300 mg of PPFMA was dissolved in 4 mL abs dioxane and 1 mL abs DMSO. A light red solution was obtained. In a typical reaction for the 50,000 g/mol precursor and 200 mg of 2-hydroxypropylamine and 200 mg triethylamine was added. The reaction was left at 50 °C overnight. The solution was concentrated under vacuum and was column filtrated using SephadexTM LH-20 in dioxane and then precipitated in diethyl ether. Thesolid was isolated by centrifugation and dried under vacuum at 30 °C for 14 h. Yield: (86%). ^{1}H NMR (DMSO-d_6): δ (ppm) 7.3–6.9 (br), 4.9–4.5 (br), 3.4–3.9 (br), 2.6–3.0, 0.9–1.5 (br).
31. Postpolymerization modification of block copolymers (**4**). In a typical reaction 300 mg of poly(PFMA)-block-poly(lauryl methacrylate) were dissolved in 4 mL abs dioxane and 1 mL abs DMSO. A slightly reddish solution was obtained. In a typical reaction 200 mg of 2-hydroxypropylamine and 200 mg triethylamine were added. The reaction was left at 50 °C over night. The solution was concentrated in vacuum and introduced to a column filtration using SephadexTM LH-20 in dioxane/DMSO (4:1) and precipitated in diethyl ether, removed by centrifugation and dried in vacuum at 30 °C for 14 h. Yield: (81%). ^{1}H NMR (DMSO-d_6): δ (ppm) 7.3–6.9 (br), 4.9–4.5 (br), 3.4–3.9 (br), 2.6–3.0 (br), 0.9–1.5 (br), 0.8–0.9 (br t).
32. Synthesis of random copolymers by postpolymerization modification (**5**). In a typical reaction 300 mg of PPFMA was dissolved in 3.5 mL abs dioxane and 1 mL abs DMSO. A light red solution was obtained. In a typical reaction for the 50,000 g/mol precursor 15 mg methyldisulfanyl cysteamin in 0.5 mL abs dioxane and 50 mg triethylamine were added. The reaction was left for 4 h at 50 °C. Afterwards 300 mg of 2-hydroxypropylamine and 300 mg triethylamine were added. The reaction was allowed to proceed at 50 °C over night. The solution was concentrated under vacuum and introduced to a column filtration using SephadexTM LH-20 in dioxane and precipitated in diethyl ether, removed by centrifugation and dried in vacuum at 30 °C for 14 h. Yield: (86%). ^{1}H NMR (DMSO-d_6): δ (ppm) 7.3–6.9 (br), 4.9–4.5 (br), 3.4–3.9 (br), 2.6–3.0, 2.4 (br), 0.9–1.5 (br).
33. Jahn, M.; Radchenko, V.; Filosofov, D.; Hauser, H.; Eisenhut, M.; Rösch, F.; Jennewein, M. *Radiochim. Acta*, in press.