Solubilisation of [60]fullerenes using block copolymers and evaluation of their photodynamic activities†

Motofusa Akiyama,[a] Atsushi Ikeda,*[a] Takashi Shintani,[a] Yuki Doi,[a] Jun-ichi Kikuchi,[a] Takuya Ogawa,[b] Keiichiro Yogo,[b] Tatsuo Takeya[b] and Naoyuki Yamamoto[c]

Received 20th December 2007, Accepted 29th January 2008
First published as an Advance Article on the web 18th February 2008
DOI: 10.1039/b719671g

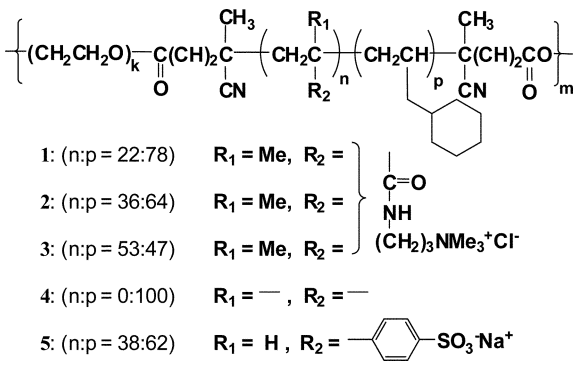
Unmodified [60]fullerenes (C_{60}) were solubilised with high stability using various type of poly(ethylene glycol) (PEG) based block copolymer micelles. Block copolymer micelle-incorporated C_{60} fullerenes were studied in cultures for biological activities using human cervical cancer HeLa cells. As a result, the cationic block copolymer micelles delivered C_{60} into the cells depending on their surface densities and showed cytotoxicity under photoirradiation.

Introduction

Photodynamic therapy (PDT) has been developed as a cancer therapy over the last 30 years and has been expanded as an emerging modality for the treatment of a variety of cardiovascular, dermatological and ophthalmic diseases.[1] Although most photosensitising drugs (PSs) currently under clinical evaluation for PDT are porphyrins or porphyrin-based molecules,[2] fullerenes have also attracted much attention as PSs because of their absorption of visible light and the formation of a long-lived triplet state.[3] Despite their potential, the poor water solubility of fullerenes has severely limited their use in applications. To solve this problem, several approaches have been studied, including modification of the fullerenes with water-soluble substituents[4] and solubilisation using polymers,[5] lipid membranes,[6] γ-cyclodextrin,[7,8] or calixarenes.[9] Of these methods, lipid membrane liposomes are of great interest not only as solubilising agents but also as materials for drug delivery systems (DDSs)[10] on account of their ability to accumulate within inflamed and solid tumour regions; the so-called enhanced permeability and retention (EPR) effect.[11] Previously, we reported that various types of lipid membrane-incorporated unmodified C_{60} ($LMIC_{60}$) fullerenes can be easily prepared, and $LMIC_{60}$ with a cationic surface showed high cytotoxicity under photoirradiation.[12,13] $LMIC_{60}$ can be stored for at least one week at 4°C, however, sedimentation was observed over longer periods of incubation (*ca.* one month). This stability of $LMIC_{60}$ remains a problem for their practical use in clinical treatment.

Recently, block copolymer micelles (BP micelles) with a hydrophobic core and hydrophilic shell have attracted much attention as solubilising agents for poorly soluble drugs and materials for DDSs.[14] BP micelles possess high stability both *in vitro* and *in vivo*, and are successfully used as drug carriers.[15] Usually, amphiphilic micelle-forming block copolymers include poly(ethylene glycol) (PEG) blocks as hydrophilic corona blocks.[16] The PEG polymer is inexpensive, has low toxicity and serves as an efficient steric protector of particulate delivery systems resulting in prolonged blood circulation kinetics.[14,17]

In this paper, we employed BP micelles as solubilising agents to improve the stability of unmodified C_{60} in nanoparticles. To this end, we synthesized various types of PEG-based cationic (**1–3**), neutral (**4**) and anionic (**5**) block copolymers (Scheme 1), and the block copolymer micelle-incorporated C_{60} fullerenes ($BPMIC_{60}$) of **1–5** were examined for their ability as drug carriers and PSs.



Scheme 1 Block copolymers synthesized in this work.

Results and discussion

Preparation and characterisation of $BMPIC_{60}$

$BPMIC_{60}$ fullerenes of **1–5** were prepared using a high-speed vibration mill.[8] Vigorously mixed BP and C_{60} were dissolved in an aqueous solution to produce a brown emulsion and centrifuged to remove the non-dispersed C_{60}. Concentrations of the respective components were evaluated using the absorbance of their UV-vis absorption spectra (ESI† Fig. S1). The order of C_{60} solubility was determined to be: **3** > **2** ≈ **4** ≈ **1** > **5** (ESI† Table S1: C_{60} solubilities 0.43, 0.38, 0.37, 0.35 and 0.18 mM). To characterise the $BPMIC_{60}$ fullerenes of **1–5**, size distributions and zeta potential

[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara, 630-0192, Japan. E-mail: aikeda@ms.naist.jp; Fax: +81 743 72 6099; Tel: +81 743 72 6099*
[b]*Graduate School of Biological Science, Nara Institute of Science and Technology, Ikoma, Nara, 630-0192, Japan*
[c]*Wako Pure Chemical Industries, Ltd., 6-1, Takada-cho, Amagasaki, Hyogo, 661-0963, Japan*

† Electronic supplementary information (ESI) available: UV-Vis absorption spectra, solubility and average particle size of $BPMIC_{60}$, cell% stained with PI, molecular weight and polydispersity data for BP. See DOI: 10.1039/b719671g

Table 1 Average particle sizes (nm) determined using a light scattering method at 25°C in the absence and presence of C_{60}

Polymer	Average particle size/nm	
	Before addition of C_{60}	After addition of C_{60}
1	N. D.[a]	124
2	N. D.[a]	105
3	N. D.[a]	92
4	N. D.[a]	86
5	N. D.[a]	137

[a] N. D. denotes that particle aggregation was not detected.

Table 2 Zeta potentials of $BPMIC_{60}$

Polymer	Zeta potential/mV
1	18[a]
2	25[a]
3	30[a]
4	−3[a]
5	−25[a]

[a] [NaCl] = 10 mM, [C_{60}] = 0.1 mM, 25 °C.

studies were carried out using dynamic light scattering (DLS) and electrophoretic light scattering spectrophotometry. The average diameters of all BP micelles in the presence of C_{60} were mostly in the range from 80 to 140 nm, at which size EPR effects can be expected, whereas particle aggregation was not detected in the absence of C_{60} (Table 1). As shown by the zeta potential, the surface charge of each $BPMIC_{60}$ corresponded with those of the corresponding BP elements (Table 2). The $BPMIC_{60}$ fullerenes of **1**–**5** could be stably stored for at least four months at room temperature (ESI† Table S2).

Intracellular uptake of $BPMIC_{60}$

The $BPMIC_{60}$ fullerenes of **1–5** were studied in cultures for biological activities using human cervical cancer HeLa cells. At first, we evaluated the ability of these micelles as drug carriers. To this end, the intracellular uptake of the $BPMIC_{60}$ fullerenes of **1–5** was quantified by monitoring the reduction of $BPMIC_{60}$ in the culture medium. After 24 h incubation with HeLa cells, the $BPMIC_{60}$ reduction was determined using the absorbance of 340 nm (ESI† Fig. S2). The cationic $BPMIC_{60}$ fullerenes of **1–3** delivered fullerenes into cells depending on their surface densities. On the other hand, no intracellular uptake of fullerenes was monitored when the anionic and neutral $BPMIC_{60}$ fullerenes of **4** and **5** were incubated with cells. The effects of the surface densities were consistent with those described in previous reports.[13,18]

Photodynamic activity of $BPMIC_{60}$

We then examined the photodynamic activity of $BPMIC_{60}$. To visualize nonviable cells, propidium iodide (PI) was used because the nonviable cells cannot exclude PI. After 24 h incubation with $BPMIC_{60}$ ([C_{60}] = 2.5 μM), the cells were exposed to light (350–500 nm, 19 mW cm^{-2}) for 2 h each. Cells treated with the $BPMIC_{60}$ fullerenes of **1–3** showed an abnormal shape and were stained with PI after light irradiation, though other treatments and all treatments without light irradiation induced no apparent change (Fig. 1 and 2). Furthermore, the percentages of cells stained with PI were increased by additional culturing for 24 h after photoirradiation. In all, 65.7, 87.0 and 98.7% of cells were stained with PI by treatment of the cationic $BPMIC_{60}$ fullerenes of **1–3** in combination with light irradiation (ESI† Table S3). On the other hand, few cells were stained by the neutral and anionic $BPMIC_{60}$ fullerenes of **4** and **5** (0.7% for **4** and 0.9% for **5**). These results are consistent with those for the intracellular uptake of

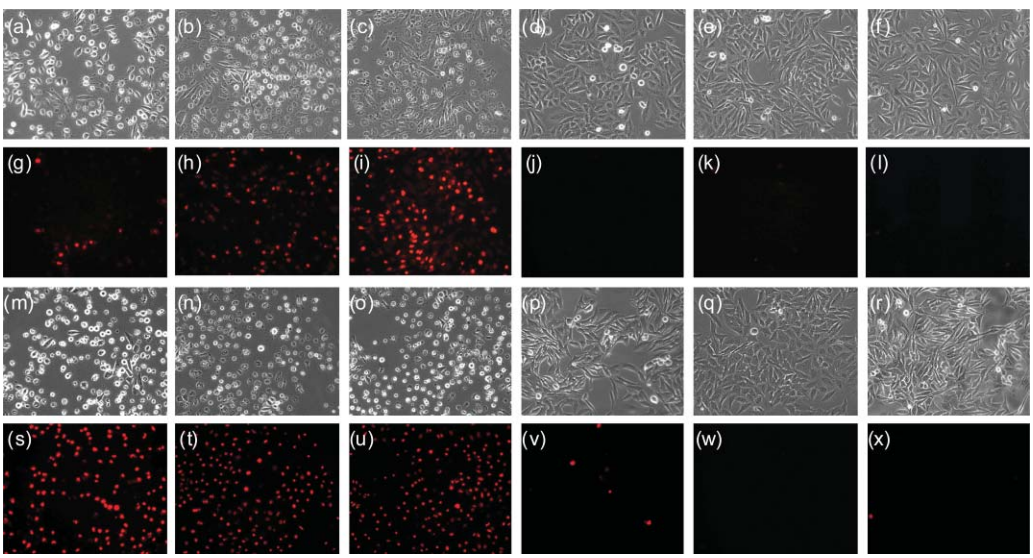


Fig. 1 Photodamage from $BPMIC_{60}$ fullerenes in HeLa cells. $BPMIC_{60}$ treated cells were exposed to light (350–500 nm) for 2 h at 35 °C (a–e, g–k, m–q, s–w) or incubated at 35 °C without light irradiation as a control (f, l, r and x). Following light irradiation (a–l) and 24 h incubation after light irradiation (m–x), the cells were stained with PI and observed using fluorescence microscopy: phase contrast images (a–f, m–r), PI (g–l, s–x). (a), (g), (m) and (s): $BPMIC_{60}$ of **1**, (b), (h), (n) and (t): $BPMIC_{60}$ of **2**, (c), (f), (i), (l), (o), (r), (u) and (x): $BPMIC_{60}$ of **3**, (d), (j), (p) and (v): $BPMIC_{60}$ of **4** and (e), (k), (q) and (w): $BPMIC_{60}$ of **5**.

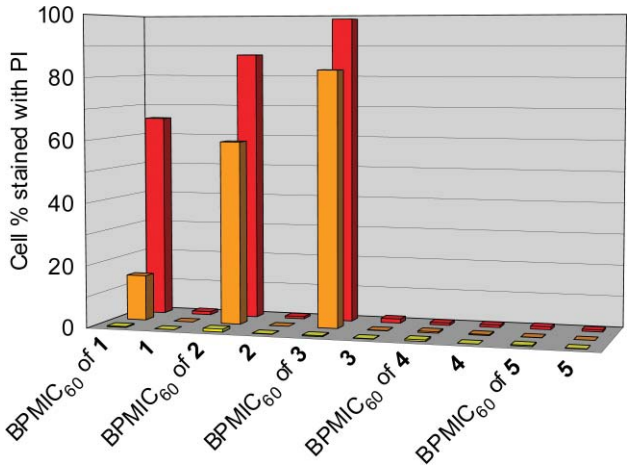


Fig. 2 Cell percentages stained with PI of HeLa cells treated with the $BPMIC_{60}$ fullerenes of **1–5** and BP alone as a control; HeLa cells without light irradiation (yellow bars), after light irradiation for 2 h at 35 °C and without incubation (orange bars) and after light irradiation for 2 h at 35 °C and incubation in the dark for 24 h at 37 °C (red bars). Each experiment was repeated three times; the average is shown.

$BPMIC_{60}$, indicating that intracellular $BPMIC_{60}$fullerenes can act as a photosensitiser.

Analysis of the mode of cell death induced by $BPMIC_{60}$

It is known that early apoptotic cells can exclude PI, such that its ability will be lost depending on the progression of apoptosis.[19] Since this phenomenon resembles the increase in percentages of cells stained with PI in cationic $BPMIC_{60}$ treatments after photoirradiation, we analysed whether apoptotic cell death was induced by cationic $BPMIC_{60}$ fullerenes. To this end, cell surface-exposed phosphatidyl serine, which occurred in the earlier stages of apoptosis, was visualised using FITC-conjugated annexin V. By combining FITC-conjugated annexin V with PI, the different labelling patterns identified the different cell fractions, *i.e.* PI-negative/annexin V-negative viable cells, PI-negative/annexin V-positive early apoptotic cells, PI-positive/annexin V-positive late apoptotic and/or necrotic cells. As a result, several cells, especially those treated with the $BPMIC_{60}$ fullerenes of **1**, were stained with only annexin V after light irradiation, indicating that these cells undergo early apoptosis (Fig. 3), whereas all nonviable cells were stained with not only PI but also annexin V after 24 h exposure to light irradiation. These results suggest that cationic $BPMIC_{60}$ fullerenes have the potential to induce apoptosis when combined with light irradiation.

Conclusions

In this study, five kinds of BPs were synthesized and their corresponding BP micelles shown to have the ability to act as solubilising agents of unmodified C_{60}. These solubilised C_{60} fullerenes were stably stored for at least four months, while the $BPMIC_{60}$ fullerenes were kept at room temperature. This stability of the unmodified C_{60} in nanoparticles may extend its application. Furthermore, the cationic $BPMIC_{60}$ fullerenes of **1–3** showed cytotoxicity that depended on their ability to act as drug carriers under photoirradiation, whereas no cytotoxicity was monitored before photoirradiation. These findings imply, therefore, that the concepts of cultivated BP micelle-incorporated C_{60} and their homologues can be more fruitfully applied to medicinal chemistry.

Experimental

General

UV-Vis spectra were obtained on a UV-2550 spectrophotometer (Shimadzu Corporation). Size distributions and zeta potentials were measured using a DLS-6000HL (Otsuka Electronics Co. Ltd.) and an ELS-3000N (Otsuka Electronics Co. Ltd.). Light irradiation was performed using a xenon lamp (MAX-301, 300 W; Asahi Spectra Co. Ltd.) equipped with a purpose-built mirror module (350–500 nm; Asahi Spectra Co. Ltd.) and a long-pass filter with cut-off at 350 nm (Asahi Spectra Co. Ltd.). Fluorescence microscopy was performed with an inverted Axiovert 135M (Carl Zeiss Inc.), equipped with an AxioCam CCD camera (Carl Zeiss Inc.) and AxioVision 3.0 software (Carl Zeiss Inc.). The following sets of filters were used: PI (BP546, FT580, and LP 590); and FITC (450–490, FT 510, and 515–565).

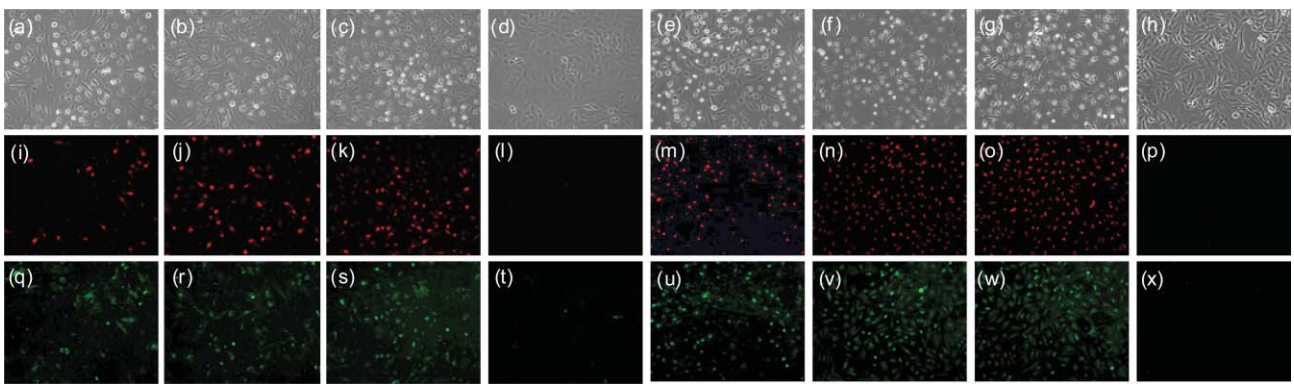


Fig. 3 Analysis of the mode of cell death induced by cationic $BPMIC_{60}$ fullerenes in HeLa cells. Following light irradiation (a–d, i–l, q–t) and 24 h incubation after light irradiation (e–h, m–p, u–x), the cells were stained with both PI and FITC-conjugated annexin V and observed using fluorescence microscopy: phase contrast images (a–h), PI (i–p) and annexin V (q–x). (a), (e), (i), (m), (q) and (u): $BPMIC_{60}$ of **1**, (b), (e), (j), (m), (r) and (v): $BPMIC_{60}$ of **2**, (c), (g), (k), (o), (s) and (w): $BPMIC_{60}$ of **3** and (d), (h), (l), (p), (t) and (x): $BPMIC_{60}$ of **4**.

Table 3 Structure and stoichiometry of monomers A and B

BP	Monomer A	Grams	Monomer B	Grams
1	3-Methacrylamido-*N,N,N*-trimethylpropan-1-aminium chloride	5.0	Allylcyclohexane	10.0
2	3-Methacrylamido-*N,N,N*-trimethylpropan-1-aminium chloride	7.5	Allylcyclohexane	7.5
3	3-Methacrylamido-*N,N,N*-trimethylpropan-1-aminium chloride	10.0	Allylcyclohexane	5.0
4	—	0.0	Allylcyclohexane	5.0
5	Sodium 4-vinylbenzenesulfonate	7.5	Allylcyclohexane	7.5

Preparation of BPs

A solution of monomer A and monomer B (Table 3) in 2-propanol (150 ml) was added to VPE-0201 (5.0 g; macro azo initiator containing PEG segments manufactured by Wako Pure Chemical Industries, Ltd.; ESI† Scheme S1) and the mixture was stirred at 78 °C under an argon atmosphere for 6 h. The mixture was poured into 500 ml hexane and the supernatant was removed through decantation. The residue was dried to give the desired BP.

Solubilisation of C_{60} using block copolymer

Mixtures of the BP (2.00 mg) and C_{60} (1.00 mg; MER Co.) were placed in an agate capsule together with two agate mixing balls and vigorously mixed at 1800 rpm for 20 min using a high-speed vibration mill (MM200; Retsch Co. Ltd.). The solid mixtures were dissolved in 3.6 ml water to produce a brown emulsion. To remove the non-dispersed C_{60}, the solutions were centrifuged at 18000*g* for 10 min at 20 °C and the resulting supernatants collected. The resulting solutions were subjected to UV-Vis spectroscopic measurements using a 1 mm cell. Concentrations of C_{60} were determined using a molar extinction coefficient for the aggregation state of C_{60}·γ-CDx complex of $\varepsilon_{340} = 2.0 \times 10^4$ M^{-1} cm^{-1}.

Cell culture

HeLa cells were maintained in CO_2 Independent Medium (Gibco BRL) supplemented with 10% foetal calf serum at 37 °C in 5% CO_2. For experiments to determine the biological activities of the $BPMIC_{60}$ fullerenes, the cells were seeded on 35 mm plates at a density of 3.4×10^5 cells $plate^{-1}$. After growing overnight, the cells were used for the experiments.

Analysis of intracellular uptake

The cells were incubated with $[C_{60}] = 2.5$ μM of $BPMIC_{60}$ for 24 h in the dark at 37 °C in 5% CO_2. After 24 h incubation, the culture medium was collected and centrifuged at 1000*g* for 5 min at 20 °C to remove the floating cells. The reduction of $BPMIC_{60}$ in the culture medium was determined by monitoring the absorbance at 340 nm (10 mm cell). The concentrations of C_{60} were determined using $\varepsilon_{340} = 2.0 \times 10^4$ M^{-1} cm^{-1}. The culture medium collected from $BPMIC_{60}$ non-treated cells was used as a blank. The non-specific reduction of $BPMIC_{60}$ in culture medium was not monitored when $BPMIC_{60}$ was incubated without cells on the culture plate for 24 h.

Photodynamic activity experiments

The cells treated with $BPMIC_{60}$ were washed with phosphate-buffered saline (PBS) and exposed to light followed by washing with fresh medium. Photoirradiation was carried out under a 19 mW cm^{-2} light power at the cell level for 2 h at 35°C. To visualize the nonviable cells, the cells were stained with PI (1 μg ml^{-1}; Sigma-Aldrich) for 10 min at room temperature following $BPMIC_{60}$ treatment, photoirradiation and 24 h incubation after photoirradiation, respectively. After washing with PBS, the cells were replaced with fresh medium and monitored by fluorescence microscopy.

Cell staining with PI and FITC-conjugated annexin V

For analysis of the mode of cell death induced by cationic $BPMIC_{60}$, the cells were seeded on glass coverslips. After photoirradiation, the cells were stained with PI (1 μg ml^{-1}) and FITC-conjugated annexin V (0.5 μg ml^{-1}; BioVision) according to the manufacturer's instructions. After staining with PI and annexin V, the cells were mounted in a Permafluor (Beckman Coulter Inc.) apparatus and monitored by fluorescence microscopy.

Notes and references

1 T. J. Dougherty, C. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng, *J. Natl. Cancer Inst.*, 1998, **90**, 889; H. Kato, *J. Photochem. Photobiol., B: Biol.*, 1998, **42**, 96.
2 K. Lang, J. Mosinger and D. M. Wagnerová, *Coord. Chem. Rev.*, 2004, **248**, 321 and references therein.
3 Y. Tabata and Y. Ikada, *Pure Appl. Chem.*, 1999, **71**, 2047; F. Rancan, M. Helmreich, A. Mölich, N. Jux, A. Hirsch, B. Röde, C. Witt and F. Böhm, *J. Photochem. Photobiol., B: Biol.*, 2005, **80**, 1; M. E. Milanesio, M. G. Alvarez, V. Rivarola, J. J. Silber and E. N. Durantini, *Photochem. Photobiol.*, 2005, **81**, 891; C. Yu, T. Canteenwala, L. Y. Chiang, B. Wilson and K. Pritzker, *Synth. Met.*, 2005, **153**, 37.
4 T. D. Ros and M. Prato, *Chem. Commun.*, 1999, 663; E. Nakamura and H. Isobe, *Acc. Chem. Res.*, 2003, **36**, 807 and references therein.
5 Y. N. Yamakoshi, T. Yagami, K. Fukuhara, S. Sueyoshi and N. Miyata, *J. Chem. Soc., Chem. Commun.*, 1994, 517.
6 H. Hungerbühler, D. M. Guldi and K.-D. Asmus, *J. Am. Chem. Soc.*, 1993, **115**, 3386; R. V. Bensasson, E. Bienvenue, M. Dellinger, S. Leach and P. Seta, *J. Phys. Chem.*, 1994, **98**, 3492.
7 T. Andersson, K. Nilsson, M. Sundahl, G. Westman and O. Wennerström, *J. Chem. Soc., Chem. Commun.*, 1992, 604.
8 K. Komatsu, K. Fujiwara, Y. Murata and T. Braun, *J. Chem. Soc., Perkin Trans. 1*, 1999, 2963.
9 A. Ikeda, T. Hatano, M. Kawaguchi, H. Suenaga and S. Shinkai, *Chem. Commun.*, 1999, 1403; T. Hatano, A. Ikeda, M. Sano, Y. Kanekiyo and S. Shinkai, *J. Chem. Soc., Perkin Trans. 2*, 2000, 909; A. Ikeda, T. Hatano, S. Shinkai, T. Akiyama and S. Yamada, *J. Am. Chem. Soc.*, 2001, **123**, 4855; A. Ikeda, T. Hatano, T. Konishi and S. Shinkai, *Tetrahedron*, 2003, **59**, 3537; A. Ikeda, T. Hatano, T. Konishi, J. Kikuchi and S. Shinkai, *Tetrahedron*, 2003, **59**, 3537; T. Konishi, A. Ikeda, M. Asai, T. Hatano, S. Shinkai, M. Fujitsuka, O. Ito, Y. Tsuchiya and J. Kikuchi, *J. Phys. Chem. B*, 2003, **107**, 11261; A. Ikeda, A. Ejima, K. Nishiguchi, J. Kikuchi, T. Matsumoto, T. Hatano, S. Shinkai and M. Goto, *Chem. Lett.*, 2005, **34**, 308.
10 Y. Kaneda, *Adv. Drug Delivery Rev.*, 2000, **43**, 197; V. P. Torchilin, *Nat. Rev. Drug Discovery*, 2005, **4**, 145 and references therein.
11 Y. Matsumura and H. Maeda, *Cancer Res.*, 1986, **46**, 6387; H. Maeda, L. Seymour and Y. Miyamoto, *Bioconjugate Chem.*, 1992, **3**, 351.
12 A. Ikeda, T. Sato, K. Kitamura, K. Nishiguchi, Y. Sasaki, J. Kikuchi, T. Ogawa, K. Yogo and T. Takeya, *Org. Biomol. Chem.*, 2005, **3**, 2907.
13 A. Ikeda, Y. Doi, K. Nishiguchi, K. Kitamura, M. Hashizume, J. Kikuchi, K. Yogo, T. Ogawa and T. Takeya, *Org. Biomol. Chem.*, 2007, **5**, 1158.
14 N. Nishiyama and K. Kataoka, *Pharmacol. Ther.*, 2006, **112**, 630; V. P. Torchilin, *Pharm. Res.*, 2007, **24**, 1 and references therein.

15 S. Danson, D. Ferry, V. Alakhov, J. Margison, D. Kerr, D. Jowle, M. Brampton, G. Halbert and M. Ranson, *Br. J. Cancer*, 2004, **90**, 2085; Y. Matsumura, T. Hamaguchi, T. Ura, K. Muro, Y. Yamada, Y. Shimada, K. Shirao, T. Okusaka, H. Ueno, M. Ikeda and N. Watanabe, *Br. J. Cancer*, 2004, **91**, 1775; T. Y. Kim, D. W. Kim, J. Y. Chung, S. G. Shin, S. C. Kim, D. S. Heo, N. K. Kim and Y. J. Bang, *Clin. Cancer Res.*, 2004, **10**, 3708.
16 G. S. Kwon, *Crit. Rev. Ther. Drug Carrier Syst.*, 2003, **20**, 357 and references therein.
17 A. L. Klibanov, K. Maruyama, V. P. Torchilin and L. Huang, *FEBS Lett.*, 1990, **268**, 235; P. Calvo, B. Gouritin, I. Brigger, C. Lasmezas, J. Deslys, A. Williams, J. P. Andreux, D. Dormont and P. Couvreur, *J. Neurosci. Methods*, 2001, **111**, 151; S. M. Moghimi, *Biochim. Biophys. Acta*, 1999, **1999**, 2963.
18 C. R. Miller, B. Bondurant, S. D. McLean, K. A. McGovern and D. F. O'Brien, *Biochemistry*, 1998, **37**, 12875.
19 M. V. Engeland, L. J. Nieland, F. C. Ramaekers, B. Schutte and C. P. Reutelingsperger, *Cytometry*, 1998, **31**, 1.