Electronic Supplementary Material (ESI) for Nanoscale Advances.

HER-2 targeted boron neutron capture therapy using antibody conjugated boron nitride nanotube/β-1,3-glucan complex†

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Supporting Information

Materials, cell culture, and animals

β-1,3-Glucan was used after purification from black yeast (Aureobasidium pullulans). Boron nitride tube was purchased from NAiEEL Technology Co., Ltd (Daejeon, Korea). Dimethyl sulfoxide (DMSO), methanol, acetone, *n*-hexane, ethyl acetate, dioxane, D-(-)-Fructose, $NaHCO_3$ and $MgSO_4$ were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). 3-Amino-phenol, cyanuric acid, 4-amino-1-naphthol hydrochloride and 7-aminoheptanoic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 4-Boronophenylalanine (BPA) was purchased from Sigma-Aldrich (St. Louis, MO). The cell counting kit-8 was purchased from Dojindo (Kumamoto, Japan). Rhodamine B labeled β-glucan was synthesized as reported previously [1]. Anti-ErbB2 / HER2 antibody was purchased from Abcam (Cambridge, MA, USA). Human ovarian cancer (SK-OV-3) cells and murine fibroblast (L929) cells were kindly supplied by Prof. Nagasaki (Osaka City University). These cells were maintained in Dulbecco's Modified Essential Medium (DMEM, Thermo Fischer Science, Massachusetts, USA) containing 10% fatal of fetal bovine serum (GE Healthcare, Illinois, USA) and 1% of antibiotic-antimycotic (Nacalai Tesque, Inc. Kyoto, Japan). Balb/c nu/nu mice were purchased from Japan SLC (Shizuoka, Japan).

Synthesis of 2-(3-aminophenol)-4,6-dichloro-s-triazine [2]

3-amino-phenol (1.48 g) was dissolved in acetone (20 mL). This solution was added dropwise to a suspension made by pouring cold water (40 mL) into an acetone solution of cyanuric acid chloride (20 mL) under stirring in an ice bath. A solution of $NaHCO_3$ (1.14 g) in water (13 mL) was added and the mixture was additionally stirred for 1.5 h. Acetone was removed in vacuo and the precipitate was filtered and washed cold water to give a white powder.

Synthesis of 2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-s-triazine (PAM) [2]

A solution of 4-amino-1-naphthol hydrochloride (513 mg) in acetone (3.3 mL) and water (3.3 mL) was naturized by adding $NaHCO_3$ (220 mg) in water (3.3 mL). the mixture was poured into a solution of 2-(3-aminophenol)-4,6-dichloro-s-triazinee (613 mg) in acetone (8.3 mL) and stirred at 45 ^oC for 5 h. A solution of $NaHCO_3$ (200 mg) in water (1.7 mL) was added to maintain the pH between 6 and 7. The reaction

mixture was concentrated in vacuo and the residue was purified by silica gel chromatography (hexane/ethyl acetate v/v = 3:2) to give a brown solid.

Synthesis of PAM COOH

PAM (100 mg) and 7-aminoheptanoic acid (52 mg) was dissolved in dioxane (8 mL) and water (2 mL) and refluxed at 100 ^{o}C for 24 h. The solvent was evaporated, and the residue was extracted with $CHCl_3$. The organic layer was washed with water and then drayed over MgSO4. The solvent was evaporated to give crude PAM carboxylic acid as a black-brown solid. The product was used for modification to polysaccharides without further purification. HRMS (ESI): Calcd. For $C_{26}H_{28}N_6O_4$ $[M+H]^+$ m/z 489.22448, found m/z 489.22440.

Synthesis of PAM-modified β-glucan (β-glucan-PAM)

Dried rhodamine labeled β-glucan (20 mg) and PAM-COOH (0.7 mg) were dissolved in dry DMSO (0.6 mL), and the mixture was stirred at 60 ^{o}C for 30 min. *N,N′*-dicyclohexylcarbodiimide (0.6 mg) and 4-dimethylaminopyridine (0.8 mg) in DMSO (0.2 mL) were added to the mixture, and stirred at 45 ^{o}C. After being stirred for 48h, the reaction mixture was dialyzed for 1 day against DMSO and 2 days against water using a Spectra/Por 6 with a molecular weight cut-off 8.0 kDa. After dialysis, a solvent was lyophilized, and the degrees of substitution were determined by ^{1}H NMR spectra.

Preparation of BNNT/β-glucan and BNNT/β-glucan-PAM complex *via* HSVM

BNNT (1 mg) and β-glucan or β-glucan-PAM (10 mg) were mixed in a vial and treated with HSVM (25 Hz, 30 min). The resulting mixture was dispersed in Milli-Q (1 mL). To remove the precipitates, the sample was centrifuged at 4500 rpm for 20 min. The supernatant was filtered through Advantec filter (pore size 0.45 μm). The Boron concentration was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Vista-MPX, Seiko instruments Inc., Tokyo, Japan).

Preparation of BNNT/β-glucan-IgG complex

The BNNT/β-glucan-PAM complex suspension and antibody solution were mixed at a 1:5 molar ratio of PAM and antibody (IgG) for 24 hours at room temperature. The FRET was determined using a fluorometer (F-4500 fluorescence spectrophotometer; Hitachi Ltd., Tokyo, Japan) with Rhodamine isothiocyanate-

conjugated β-glucan-PAM and fluorescein isothiocyanate-conjugated antibody. BNNT/β-glucan-IgG complex was excited at 495 nm and the emission intensity was measured from 500 nm to 650 nm.

Characteristics of BNNT/β-glucan, BNNT/β-glucan-PAM and BNNT/β-glucan-IgG complex

The hydrodynamic diameter (D_{hy}) and ζ-potential measurements were carried out with a Zetasizer Nano ZS (Malvern, Malvern, UK). The morphology was performed at an accelerating voltage of 80 kV (JEM-1400 field emission electron microscope, JEOL Ltd., Tokyo, Japan). Specimens were prepared by casting a sample solution on an ultrathin carbon-deposited Cu grid and staining with 1% of phosphotungstic acid.

Cytotoxicity Assay of BNNT/β-glucan complex and BNNT/β-glucan-IgG complex

L929 cells were seeded on 96-well plate at a density of 5.0×10^3 cells/well and incubated overnight. The cells were treated with BNNT/β-glucan complex at varying concentrations. After 24h incubation, a Cell counting Kit-8 solution was added to each well and plates were further incubated for 2h. After incubation, absorbance at 450 nm was measured using a microplate reader. The cytotoxicity of the BNNT/β-glucan-IgG complex was similarly examined using SK-OV-3 cells.

Quantification of the cellular uptake of boron and evaluation of the cellular uptake mechanism

SK-OV-3 cells were seeded on 6 well plate at a density of 1.0×10^6 cells/well and incubated overnight. Next, the cells were exposed to the BNNT/β-glucan and BNNT/β-glucan-IgG complex. The final Boron concentrations was adjusted to 1 ppm and 10 ppm. The cells were collected and lysed with a mixture of nitric acid and hydrochloric acid (v/v = 1:3), followed by heating at 90 ^{o}C for 0.5 h and 115 ^{o}C for 1.5 h. The ashed sample was diluted with water, and the volume was adjusted to 5 mL, followed by centrifugation (1500 rpm, 25 °C, 5 min) to remove the precipitates. The amount of boron uptake in the cells was determined using ICP-AES.

For the HER-2 inhibition, Anti-HER-2 antibody was applied for 4 h at 0 to 2 equivalents against PAM before the cells were treated with BNNT/β-glucan-IgG complex.

Subcellar distribution of BNNT/β-glucan and BNNT/β-glucan-IgG complex

SK-OV-3 cells were seeded on glass bottom dishes at a density of 1×10^5 cells/well and incubated overnight. The cells were treated with BNNT/β-glucan or BNNT/β-glucan-IgG complex for 24 h. The cells were washed with PBS thrice. Lysosomes were stained using commercially available fluorescent reagents, Lysotracker green. The samples were observed by CLSM (LSM700, Carl Zeiss, Germany).

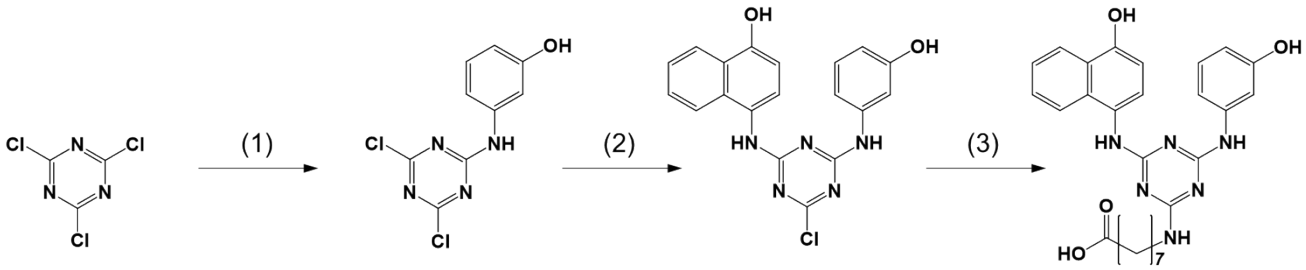
For the HER-2 inhibition, Anti-HER-2 antibody was applied for 4 h at 0.5 equivalents against PAM before the cells were treated with BNNT/β-glucan-IgG complex.

BNCT on the SK-OV-3 cells

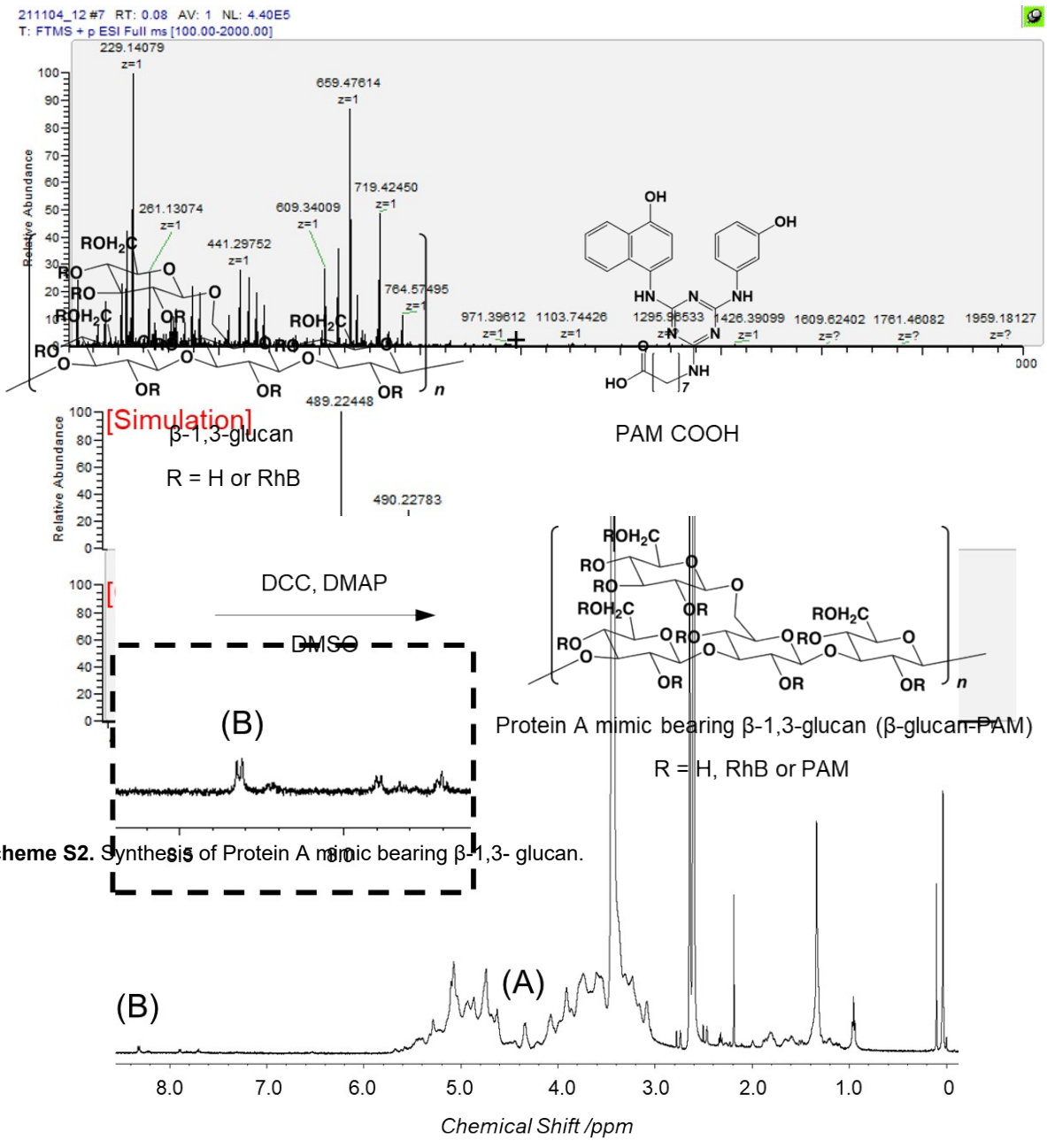
The cells were seeded on 12 well plate at a density of 1×10^5 cells/well and incubated overnight. The cells were exposed to BNNT/β-glucan complex, BNNT/β-glucan-IgG complex, BPA-fructose at a varying concentration. After 24 h incubation, the cells were exposed to thermal neutron irradiation with a fluence of 6.3×10^{11} n•cm^{-2}. The cell viabilities were evaluated after additional incubation for 24 h and 48 h.

Biodistribution of BNNT/β-glucan-IgG complex

Tumor-bearing mice were obtained by injecting a SK-OV-3 cell (5.0×10^5 cells/50 μL) suspension in HBSS at the right femur of nude mice (Balb/c nu/nu, Japan SLC). After 30 days incubation, the BNNT/β-glucan-IgG complex ([B] = 200 ppm, mice 100μL) were intravenously injected. At 3, 6 and 24h post injection, tumors, blood, and organs (kidney, lung liver normal (skin), brain, spleen, and heart) were collected. The collected samples were soaked in aqua regia and ashed at 115 ^{o}C. The resulting solution was analyzed using ICP-AES (n = 3).



Scheme S1. Synthesis of PAM-COOH. (1) 3-amino phenol, $NaHCO_3$, acetone, water. (2) 4-amino-1-naphthol hydrochloride, $NaHCO_3$, acetone, water. (3) 7-aminoheptanoic acid, dioxane, water.



Scheme S2. Synthesis of Protein A mimic bearing β-1,3- glucan.

Fig. S2. ^{1}H-NMR spectra of β-glucan-PAM in d_6-DMSO. (A) anomeric proton in β-glucan and (B) naphthyl proton of PAM

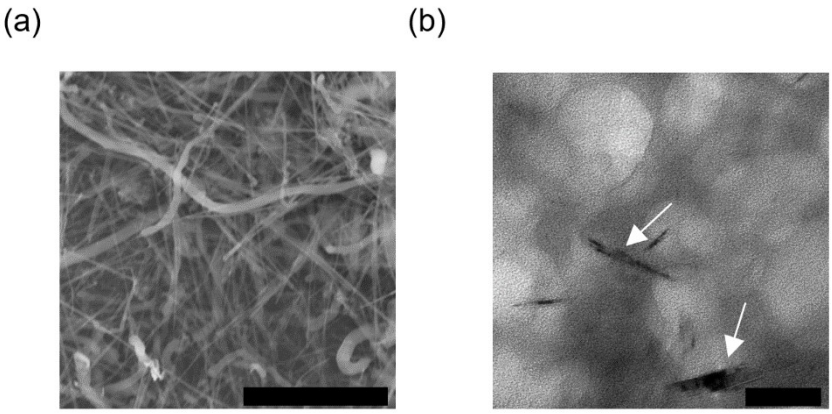


Fig. S3. (a) SEM image of BNNT. Scale bar = 2.5 μm. (b) TEM image of BNNT/β-glucan-IgG complex. White arrows indicate the bundle structures. Scale bar = 40 nm

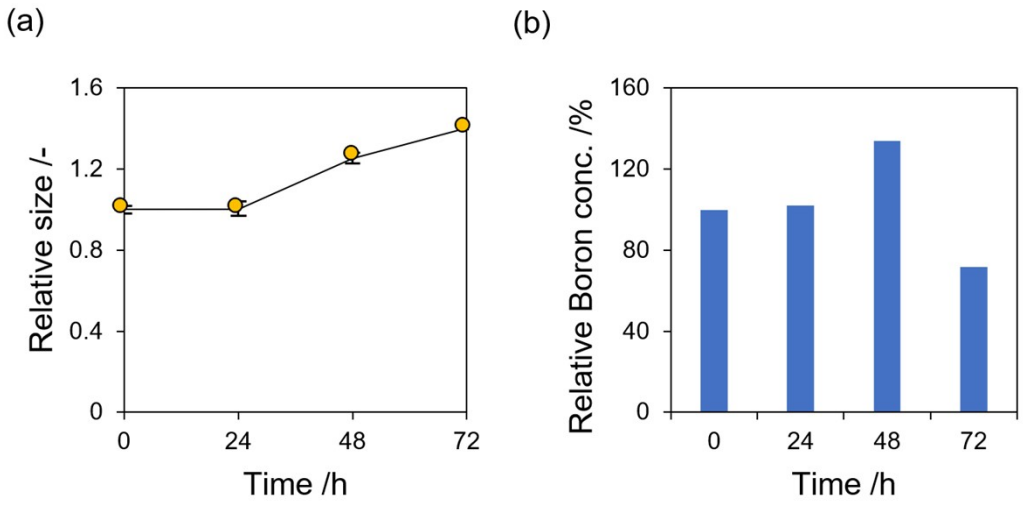


Fig. S4. Th of BNNT/β-glucan-IgG natant.

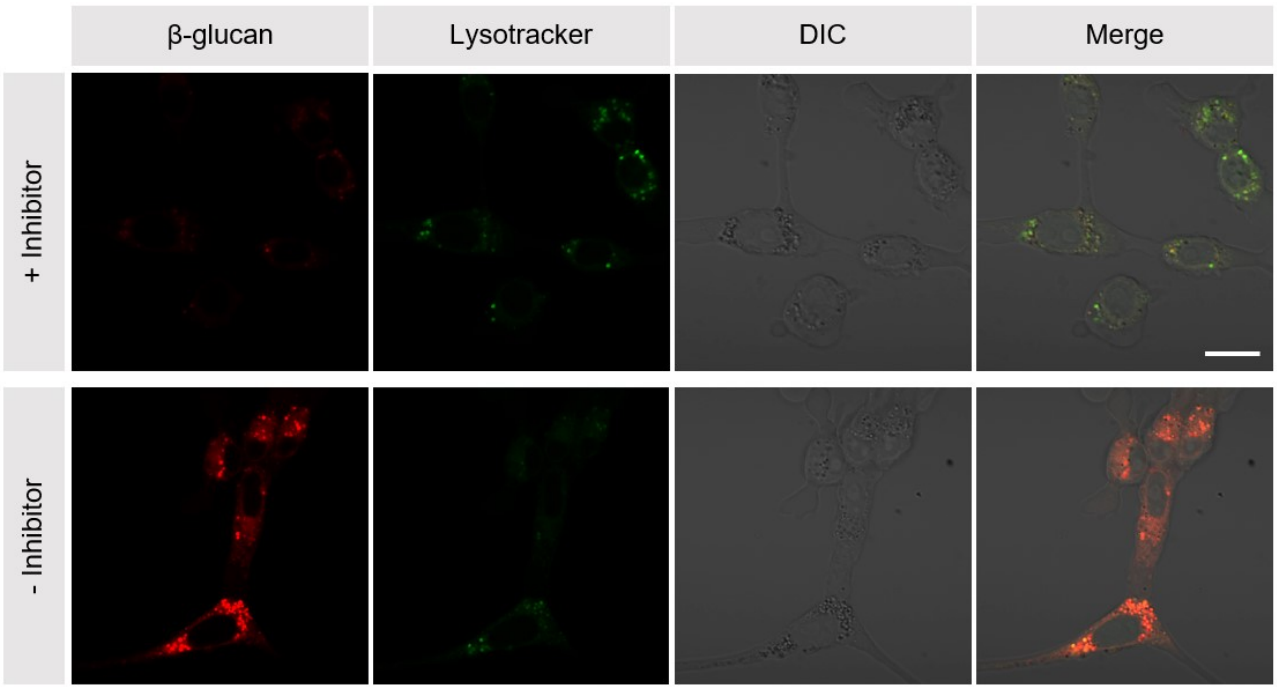


Fig. S5. Subcellular distribution of BNNT/β-glucan-IgG complex in the SK-OV-3 cells observed by confocal laser scanning microscopy. The SK-OV-3 cells were preincubated with Free HER-2 targeting antibody as the inhibitors before treated with BNNT/β-glucan-IgG complex for 24h. After 24 h of incubation with BNNT/β-glucan complex (red), lysosomes were stained with Lysotracker Green (green). Scale bar = 20 μm.

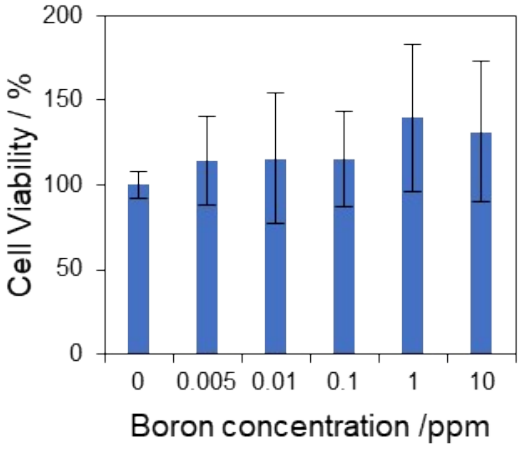


Fig. S6. Cell viability of the SK-OV-3 cells treated with BNNT/β-glucan-IgG complex in the absence of thermal neutron irradiations.

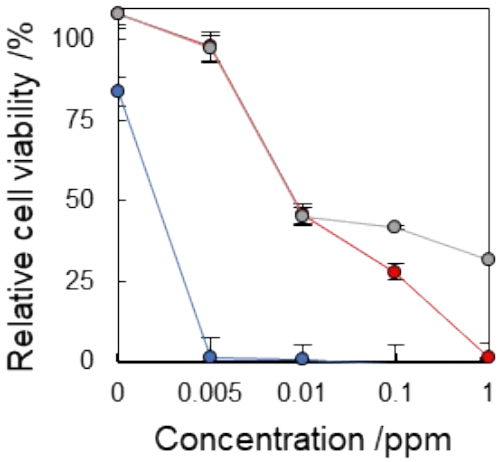


Fig. S7. The efficacy of BNCT on cell viability in SK-OV-3 cells. The cells were incubation with BNNT/β-glucan-IgG complex (blue), BNNT/β-glucan complex (red) or BPA-fructose (gray) for 24h. After incubation, the cells were exposed to thermal neutron irradiation with a fluence of 6.3×10^{11} n•cm^{-2}. The cell viabilities were evaluated after additional incubation for 48 h.

Reference

[1] Y. Tahara, SA. Mukai, S. Sawada, Y. Sasaki, and K. Akiyoshi, *Advanced Materials*, 2015, **27**, 5080-5088

[2] S. F. Teng, K. Sproule, A. Husain, and C. R. Lowe, *J. Chromatogr. B Biomed. Appl.*, 2000, 740, 1-15