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In vitro and *in vivo* anti-tumor effects of novel Span 80 vesicles containing immobilized *Eucheuma serra* agglutinin

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ABSTRACT

The lectin *Eucheuma serra* agglutinin (ESA) is known from previous studies to specifically bind to high-mannose type *N*-glycans and to induce apoptotic cancer cell death *in vitro*. In this study, Span 80 vesicles, with an average diameter between about 200 and 400 nm, containing immobilized ESA were prepared from the nonionic surfactant Span 80, also known as sorbitan monooleate. The vesicles were investigated *in vitro* and *in vivo* to evaluate the vesicles's potential applicability as novel drug delivery system. The results obtained are promising since the following was observed: (i) vesicular ESA had the same hemagglutinating activity as free ESA, demonstrating its biological activity when bound to the vesicles; (ii) vesicles containing immobilized ESA decreased the viability of Colo201 cancer cells *in vitro* while the growth of normal cells was not affected; (iii) the vesicles showed binding to Colo201 cells *in vitro* and caused inhibition of cancer cell growth in nude mice to which the vesicle-treated cells were added; (iv) the vesicles diminished tumor growth after intravenous administration to nude mice which contained an implanted Colo201 tumor; (v) the vesicles showed a tendency to accumulate at the site of the tumor 6 h after *i.v.* administration to nude mice. Thus, all measurements carried out indicate that this type of Span 80 vesicle can be considered as promising alternatives to conventional phospholipid-based vesicles.

1. Introduction

In recent years there have been numerous investigations of novel drug delivery systems (DDS) for elucidating their applicability as drug carriers for the treatment of various diseases (Allen and Cullis, 2004; Ferrari, 2005; Lian and Ho, 2001; Peer et al., 2007; Couvreur and Vauthier, 2006). Phospholipid vesicles (liposomes), i.e. vesicles composed of natural phospholipids, are often used as DDS (Lian and Ho, 2001; Sharma and Sharma, 1997; Barenholz, 2001; Torchilin, 2005), e.g. for active targeting of specific colon cancer cells (Sato et al., 1988; Koning et al., 2002; Hatziantoniou et al., 2006; Garg et al., 2009). On the other hand, it has been demonstrated that nonionic vesicles prepared from Span 80 have promising physico–chemical properties (high membrane fluidity with temperature dependent fusiogenicity) which make this type of vesicle an attractive possible alternative to the commonly used liposomes (Kato et al., 1993; Kato and Hirata, 1996; Kato and Hirashita, 1997; Ohama et al., 2005; Sugahara et al., 2005). In the food and cosmetic industries, Span 80 is generally known as sorbitan monooleate, although commercial Span 80 is a heterogeneous mixture of sorbitan mono-, di- tri, and tetraesters (Kato et al., 2006).

Span 80 vesicles can be prepared by a recently developed two-step emulsification method which yields vesicles with a membrane composition which is significantly different from commercial Span 80 (Kato et al., 2006). The bilayer membrane of Span 80 vesicles forms under thermodynamic control during the vesicle preparation, with partial elimination of those components present in commercial Span 80 which hinder formation of stable bilayers. Span 80 vesicles have rather fluid membranes; addition of soybean

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lecithin and cholesterol (at 9 and 4.5 wt%, respectively) lead to a stabilization of the membrane with a lowering of the membrane permeability (Kato et al., 2008). Furthermore, the two-step emulsification allows the preparation of vesicles with relatively high encapsulation yields for water soluble molecules by entrapping the molecules as inner phase solution, just before the first emulsification is carried out (Figure S1 in Supplementary Material, and Kato et al., 2006; Kato et al., 2008).

Tumor-specific "active targeting" is often achieved by immobilizing tumor-specific ligands such as antibodies, peptides or saccharides onto liposomal drug carrier systems (Peer et al., 2007; Torchilin, 2005; Forssen and Willis, 1998). While most tumor-specific ligands have no intrinsic anti-tumor activity, several lectins are known to possess anti-tumor activity against human cancer cells (Karasaki et al., 2001; Timoshenko et al., 2001; Wang et al., 2000). In this case targeting and anti-tumor activity are combined in one and the same molecule. One particular lectin with such "dual activity" is the lectin *Eucheuma serra* agglutinin (ESA) (Kawakubo et al., 1997). It can be extracted in the two isoforms ESA-1 and ESA-2 from marine red algae (Kawakubo et al., 1997). ESA-1 and ESA-2 have the same molar mass (27,950 g/mol) but differ in isoelectric points (pI = 4.75 for ESA-1 and pI = 4.95 for ESA-2) (Kawakubo et al., 1997). ESA-2 is specific for high-mannose type *N*-glycans (Hori et al., 2007). We have previously shown that ESA has a specific affinity to various cancer cells (specifically to the human colon cancer cell line Colo201), inducing apoptotic cell death *in vitro* (Sugahara et al., 2001) and *in vivo* (Fukuda et al., 2006).

In the work presented we have prepared Span 80 vesicles containing immobilized ESA and measured the activity of these vesicles against tumor cells *in vitro* and *in vivo*. Since ESA has a high affinity to Colo201 cells (see above), these cells were mainly chosen to investigate the tumor targeting properties of the vesicles. Different types of vesicles were prepared as reference systems and the anti-tumor activity of the different types of vesicles was compared by using a number of independent methods.

Contemporary liposomal DDS often contain poly(ethyleneglycol), PEG, immobilized onto the liposome surface. These "PEGylated liposomes", also called "stealth liposomes", generally show a decreased uptake by the reticuloendothelial system (RES), i.e. a prolonged blood circulation time, as compared to conventional liposomes (Couvreur and Vauthier, 2006; Zeisig et al., 1996). For this reason, we also prepared and used for *in vivo* studies PEGylated Span 80 vesicles. The different Span 80 vesicles prepared were as follows (Fig. 1): **CV**, "control vesicles", i.e. Span 80 vesicles without PEGylated lipids or ESA; **PV**, Span 80 vesicles containing PEGylated lipids (DSPE-PEG_{2000}); **EV**, Span 80 vesicles containing immobilized ESA; **EPV**, Span 80 vesicles containing PEGylated lipids and immobilized ESA; **EEPV**, Span 80 vesicles containing PEGylated lipids, immobilized ESA and entrapped ESA.

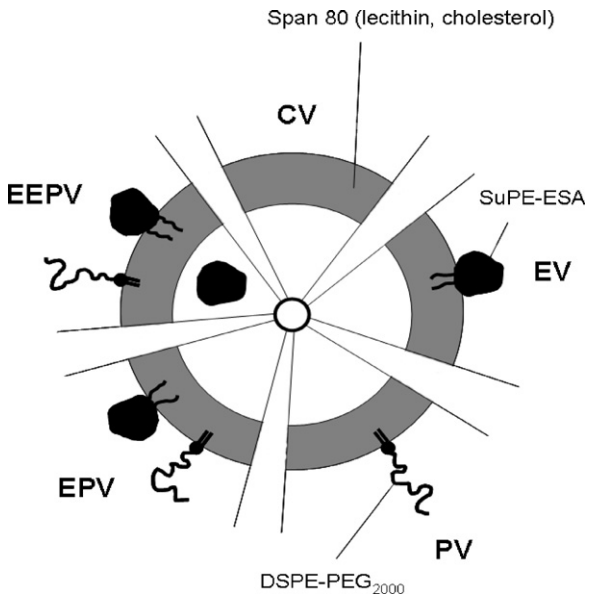

Fig. 1. Schematic representation of the different types Span 80 vesicles used. A cross section through one unilamellar Span 80 vesicle is shown in the center. The composition of the membrane of the different types of vesicles is illustrated. The highly schematic drawing is for an easier distinction of the different types of vesicles, only. The molecular details of the arrangement of the different components are not known. **CV**, Span 80 vesicles without PEGylated lipids or ESA ("control vesicles"); **PV**, Span 80 vesicles containing DSPE-PEG_{2000}; **EV**, Span 80 vesicles containing immobilized ESA; **EPV**, Span 80 vesicles containing DSPE-PEG_{2000} and immobilized ESA; **EEPV**, Span 80 vesicles containing DSPE-PEG_{2000}, immobilized ESA and entrapped ESA.

2. Materials and methods

2.1. Chemicals

Sorbitan monooleate (Span 80) and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lecithin from soybean was obtained from Wako Pure Chemical Industries (Osaka, Japan) and purified by acetone-precipitation (Inoue, 1974). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethyleneglycol)-2000] (DSPE-PEG_{2000}), which is a phospholipid to which a poly(ethyleneglycol) chain with a molar mass of 2000 g/mol is bound, was obtained from NOF Corporation (Tokyo, Japan). Cholesterol was from Wako Pure Chemical Industries.

Isothiocyanic acid octadecylester (IAOE) was synthesized from *N*,*N*-dichlorohexylcarbodiimide (DCCD) and 1-aminooctadecane as follows: In a first vessel, 3.4 g DCCD were first dissolved in 200 mL diethylether, cooled at −10 °C. 8 mL carbon disulphide was then added to this cooled solution. In a second vessel, 4.3 g 1-aminooctadecane were dissolved in 250 mL diethylether, and this solution was added to the cooled DCCD solution. The mixture was left standing at room temperature for 5 h. Afterwards, the solution was filtered using filter paper 5C (Advantec) to remove the byproduct thiourea. The filtrate was evaporated with a rotary evaporator and the obtained oily IAOE product was purified by recrystallization from diethylether.

The lectin ESA (*E. serra* agglutinin, mainly the isoform ESA-2 (Kawakubo et al., 1997) was extracted from the red alga *E. serra* and purified as described previously (Kawakubo et al., 1997). The phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-succinyl (SuPE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The radioisotope ^{125}I was obtained from MP Biomedicals Inc. (Irvine, CA, USA); ^{125}I was used to isotopically label bovine serum albumin (BSA) with 1,3,4,6-tetrachloro-3α-6α-diphenylglycouril (iodogen), obtained from Pierce Chemical Co. (Rockford, IL, USA). ^{125}I-labeled BSA was prepared as described previously (Hashizume et al., 1990). XRITC (amine-reactive X-rhodamine-5-(and-6)-isothiocyanate) was from Sigma Aldrich. All other reagents used were of guaranteed or biochemical grade.

2.2. Preparation of lipidic ESA-conjugates

The phospholipid-ESA conjugate was prepared as follows: 1 mg/mL of ESA was reacted with SuPE (1.25 mg/mL) in 0.15 M sodium carbonate buffer (pH 9.0) at room temperature. The reaction mixture was incubated for 2 h with vortexing for a few seconds every 30 min, followed by standing at 4 °C for 12 h. Residual SuPE in the buffer solution was removed by gel filtration with a PD-10 column packed with Sephadex G-25 (from Amersham Biosciences).

The IAOE-ESA conjugate was prepared as follows: 10 mg of IAOE was dissolved in 10 μL *N*,*N*-dimethyl sulfonamide (Wako Pure