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Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes

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Abstract A human anti-CD22 single chain (sc) Fv antibody fragment from a synthetic phage antibody display library was biosynthetically lipid-tagged by using *Escherichia coli* lipoprotein sequences. The purified anti-CD22 scFv lipoprotein was incorporated into liposomes by detergent dilution. Anti-CD22 immunoliposomes were shown to bind specifically in a dose- and time-dependent manner to CD22+ cell lines and CD22+ B-lymphocytes present in freshly isolated samples of blood mononuclear cells. The immunoliposomes were demonstrated to accumulate in intracellular compartments. Biosynthetically lipid-tagged human scFv antibody fragments isolated from phage display libraries may facilitate the construction of immunoliposomes with improved properties.

Key words: Phage display; Lipid-modified scFv; Immunoliposome

1. Introduction

Liposomes, spherical self-assembling colloidal particles composed of a phospholipid bilayer membrane encapsulating an internal aqueous space, have potential as carrier systems to deliver substances such as drugs, enzymes, DNA and antigens to tissues and cells. To attain specific in vivo homing, liposomes may be coated with monoclonal antibodies recognizing epitopes expressed on the target cells. A disadvantage of these 'immunoliposomes' is their rapid uptake by cells of the reticuloendothelial system (RES), possibly mediated by Fc or C3b receptors interacting with the constant region of the antibody molecule [1]. In addition, when applied in humans, immunoliposomes decorated with murine monoclonal antibodies may evoke an immune response against the foreign protein, resulting in reduced targeting efficacy and enhanced removal by cells of the RES.

Recent progress in antibody engineering and phage display technology has revolutionized our abilities to obtain antibody fragments of predefined specificities. Large repertoires of single chain (sc) Fv or Fab antibody fragments displayed on filamentous bacteriophage particles, in combination with an array of versatile phage selection procedures has yielded a broad spectrum of antibodies directed against purified and prokaryotic and eukaryotic cell-bound antigens [2,3]. Engineering steps further permit the 'tailoring' of antibody fragments to meet specific requirements in terms of avidity, pharmacokinetic properties and biological effector functions. For example, monovalent scFv fragments have been converted to bivalent and bispecific antibodies or produced as antibody-toxin fusion proteins [4].

Linking of conventional antibodies to liposomes has been achieved by chemical lipid modification of the antibodies, frequently resulting in significant protein loss, decreased activity and heterogeneity of the end product in terms of number and location of lipid moieties. Recently, a hybridoma-derived lipid-modified anti-phenyloxazolone scFv molecule has been produced in *E. coli* by genetic engineering. Bacterial lipoprotein (LPP) nucleotide sequences were fused to the DNA encoding the scFv antibody leading, upon introduction of the constructs into *E. coli*, to in vivo fatty acylation of the scFv fragment. The lipid-modified scFv purified from the bacterial membrane retained their binding activity and, upon simple removal of detergent, were shown to spontaneously, stably and quantitatively associate with liposomes in an oriented manner [5,6].

We have initiated experiments to investigate the application of phage display library-derived human scFv fragments in cell-specific targeting of liposomes. The DNA encoding a human CD22-specific scFv fragment from a large synthetic phage display library was fused to the *E. coli* LPP sequence and a hexahistidyl tag was added to facilitate purification of the modified antibody fragments. Purified scFv fragments were incorporated into immunoliposomes and specificity, binding kinetics and internalization properties were examined using cell lines and freshly isolated hematopoietic cells. The results show that lipid-modified human scFv antibody fragments from phage display libraries can be efficiently and stably incorporated into immunoliposomes. The resulting immunoliposomes specifically interact with target cells expressing the correct antigen. These experiments set the stage for the production of homogeneous preparations of potentially less immunogenic immunoliposomes for human therapy.

2. Materials and methods

2.1. scfv fragments

The anti-IgG and anti-CD22 scFv fragments were selected from a semi-synthetic antibody phage display library constructed in phagemid pHEN1. Library construction and isolation of both clones have been described in detail elsewhere [2,7].

2.2. Oligonucleotides used for cloning

lppfor: 5′ gat tac gcc aag ctt gca tgc aaa ttc tat ttc aag gag aca gtc ata atg aaa gct act aaa ctg gta ctg ggc. *lppbac*: 5′ ctg cac ctg ggc cat ggc ctg atc gat ttt agc gtt gct gga gc. *henhis*: 5′ caa acg aat gga tcc tca gtg gtg gtg gtg gtg gtg att cag atc ctc ttc tga g

2.3. Vector construction

Oligonucleotides *lppfor* and *lppbac* were used to append correct restriction sites to the *E. coli* LPP sequence. Template consisting of plasmid pTX101 [8] was polymerase chain reaction-amplified (PCR), digested with *Hin*dIII and *Nco*I and cloned into vector pHEN1 [9],

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resulting in vector pLPHEN. Another PCR reaction was performed to add a hexahistidinyl encoding sequence; pHEN1 template was amplified using primers *M13R* and the HIS-tag encoding primer *henhis*, digested with *Xho*I and *Bam*HI and cloned in vector pLPHEN, resulting in vector pLPscH. *Nco*I-*Not*I digests were performed to clone scFv encoding fragments from vector pHEN1 into vector pLPscH.

2.4. Lipoprotein-scFv expression and purification

Vector pLPscH containing the anti-CD22 or anti-IgG scFv gene were transformed to *E. coli* strain SF110 F′ [7] and plated on TYE agar containing the appropriate antibiotics and 5% glucose. Next day, cells were scraped from the plates and used to inoculate 200 ml LB medium containing 10 μg/ml ampicillin and 0.1% glucose. Cells were grown with shaking at 25°C until an OD_{600} of 0.5 was reached. IPTG was added at a final concentration of 1 mM and incubation continued overnight. Preparation of lipid-tagged scFv fragments was performed essentially as described [6]. A single IMAC step was performed to purify the scFv lipoproteins from detergent solubilized membranes. The only major deviations from the cited protocol include washing and elution of metal affinity columns in 20 mM HEPES pH 7.4, 0.5 M NaCl, 1% *n*-octyl β-D-glucoside (OG) and 10% glycerol containing 5 and 200 mM imidazole, respectively. Purity of the lipoprotein samples was visualized by SDS-PAGE followed by a Coomassie Brilliant Blue staining.

2.5. Preparation of immunoliposomes

The liposomes were composed of egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), cholesterol (Chol) and rhodamine-phosphatidylethanolamine (rhodamine) at a molar ratio of 10:1:5:0.01. EPC and EPG were gifts from Lipoid K.G., Ludwigshafen, Germany. Chol and OG were purchased from Sigma, St. Louis, USA, and rhodamine from Avanti Polar Lipids, Alabaster, USA. A mixture of the appropriate lipids in ethanol was evaporated to dryness by rotary evaporation at 35°C under reduced pressure. After flushing with nitrogen, the lipid film was solubilized in 1 ml of 4% OG containing 150 μg lipid modified scFv. After vortexing in the presence of glass beads, the mixture was diluted 26-fold in 5 min with HEPES buffer 10 mM HEPES, 135 mM NaCl, pH 7.4). The detergent was removed by collecting the immunoliposomes by ultracentrifugation (300 000 × *g* for 2 h at 4°C) and resuspension of the pellet in 5 ml of HEPES buffer. The phospholipid (PL) concentration of the final dispersion was determined to be 2 μmol/ml [10]. The mean particle size was determined by dynamic light scattering at 25°C with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Bovine serum albumin was added to a final concentration of 1%. Immunoliposomes were stored at 4°C until further use.

2.6. Binding specificity of anti-CD22 liposomes

Specificity of anti-CD22 liposomes, and as a control anti-IgG liposomes, was verified by incubating 2×10^5 cells from cell lines BJAB, Daudi, APD, BV173 (CD22+ B-lineage cells), Jurkat, K562, U266, U937 (cells from various CD22− hematopoietic lineages) or 10^6 peripheral blood leukocytes (PBL), prepared as described [2], with 100 μl liposomal stock in a total volume of 500 μl ice-cold PBS-2% BSA (PBSB). The mixtures were rotated at 4°C for 1 h and washed once in PBSB. PBLs were further incubated in 20 μl fluorescein isothiocyanate (FITC)-conjugated CD19 antibody (Becton Dickinson) for 20 min and washed again. All cells were resuspended in PBSB and analysed in a flow cytometer (Becton Dickinson) at standard settings.

2.7. Kinetics of anti-CD22 liposome binding

Binding kinetics of anti-CD22 liposomes to cell lines BJAB (a CD22+ B-cell line) and Jurkat (a CD22− T-cell line) was analysed by flow cytometry. 5×10^5 cells were mixed with increasing concentrations of immunoliposomes in a total volume of 500 μl PBSB, the mixture was slowly rotated at 4°C for 1 h, washed once in PBSB, resuspended in PBSB and analysed by flow cytometry. In a similar experiment, the immunoliposome concentration was kept constant at 200 nmol PL/ml while varying the incubation period.

2.8. Flow-cytometric analysis of anti-CD22 liposome internalization

5×10^5 cells were mixed with 100 μl immunoliposomes in a total volume of 500 μl ice-cold RPMI containing 5% fetal calf serum (RPMIS). The mixture was slowly rotated at 4°C for 1 h before adding to 1 ml RPMIS in a CO_2 incubator set at 37°C. After 0–120 min, cells were removed from the incubator, washed once in 1 ml ice-cold PBS and resuspended in 50 μl ice-cold PBS containing 15 mg/ml protease K (Qiagen, Germany). Cells were incubated on ice for 30 min, resuspended in 1 ml PBSB and analysed by flow cytometry.

2.9. Internalization of anti-CD22 liposomes; confocal laser scan microscopy

BJAB cells were mixed with anti-CD22 liposomes in RPMIS and incubated at 4°C as described previously. After this, the cells were (A) washed only, (B) treated with protease K as described, (C) incubated at 37°C for 1 h as described or (D) incubated at 37°C for 1 h before incubation with protease K. Thereafter, cells were washed twice in ice-cold PBS and mounted on Alcian Blue treated coverslips [11]. The coverslips were immersed in 3% glutaraldehyde for 30 min, washed once in PBS and fixed to slides. Cells were visualized using a confocal laser microscope (Leitz, Germany).

3. Results

3.1. Vector construction

PCR technology was used to construct vector pLPscH, adding an LPP and hexahistidyl-tag sequence to the 5′ and 3′ end, respectively, of scFv encoding genes (Fig. 1). ScFvs obtained from phage display libraries constructed with the phagemid pHEN1 can be directly cloned in this vector by a single digestion-ligation step.

3.2. Expression and purification of lipid-tagged scFv fragments

The DNA encoding previously isolated scFv antibody fragments specific for human IgG and the CD22 molecule [2,7] were cloned into vector pLPscH and the resulting constructs were introduced into *E. coli* strain SF110 for expression of the lipid-modified antibodies. Bacterial membranes were isolated by ultracentrifugation and lipid-modified scFv fragments released by extraction in Triton X-100 detergent. Immobilized metal affinity chromatography (IMAC) of these extracts repeatedly yielded ~1.5 mg lipid-tagged scFv per l of bacterial culture. Aliquots of the crude bacterial membrane extract and purified lipid-modified anti-CD22 scFv were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining of gels (Fig. 2, lane 2 and 3, respectively). A single major band of the anticipated size (approx. 28 kDa) was observed after IMAC purification.

3.3. Preparation of immunoliposomes

The lipid-modified anti-CD22 and anti-IgG scFv fragments were coupled to EPC/EPG/Chol liposomes, containing rhodamine-PE as a fluorescent bilayer marker, by dilution of mixed micelles containing OG, lipid and lipid-modified scFv to a level far below the critical micelle concentration of the detergent. Dynamic light scattering measurements yielded a mean particle diameter of approx. 0.15 μm. Incorporation of scFv molecules in the liposomes was verified by SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 2, lane 4). Mean immunoliposome particle size and immunoreactivity did not alter significantly over a 3 month storage period at 4°C (results not shown).

Fig. 1. Schematic representation of vector pLPscH containing an scFv encoding gene. The lacZ promotor-operator region (P/O), *E. coli* lipoprotein signal sequence (SS-LPP), *E. coli* lipoprotein N-terminal amino acids (LPP), scFv gene (VH-VL), Myc-tag (Myc) and hexahistidyl tag (H) are indicated.

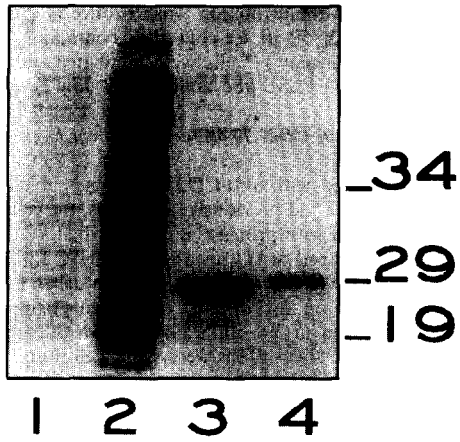

Fig. 2. Expression and purification of the lipid modified anti-CD22 scFv fragment and reconstitution into liposomes. Protein samples were run on a reducing SDS-PAGE gel and visualized by staining with Coomassie Brilliant Blue. 1, lipid modified anti-CD22 scFv expressing *E. coli* culture; 2, pelleted bacterial membranes from the aforementioned bacteria; 3, IMAC-purified bacterial membrane extract; 4, anti-CD22 scFv liposomes.

3.4. Binding specificity of anti-CD22 liposomes

Binding specificity of the immunoliposomes was initially assessed by incubation of rhodamine-labelled liposomes with a panel of CD22+ B-cell lines (BJAB, Daudi, APD and BV173) and CD22− cell lines (Jurkat, K562, U266 and U937), representing various hematopoietic lineages. The anti-CD22 liposomes displayed exclusive binding to all B-cell lines. Control anti-IgG scFv immunoliposomes did not bind to any of the cell lines. Specificity of binding was further evaluated by incubating rhodamine-labeled anti-CD22 liposomes with freshly isolated peripheral blood leukocytes. Double staining with a FITC labeled anti-CD19 monoclonal antibody was performed to identify the B-lymphocyte target population. Flow-cytometric analysis shows specific staining of virtually all CD19+ cells in the sample (Fig. 3). The immunoliposomes do not noticeably interact with cells of other hemàtopoietic lineages in the mixture. Note that the rhodamine signal is weak because the excitation/emission characteristics of this fluorochrome are not optimal for analysis on a flow cytometer equipped with a 488 nm argon-ion laser.

3.5. Binding kinetics of anti-CD22 liposomes

We evaluated the binding kinetics of rhodamine-PE-loaded anti-CD22 scFv immunoliposomes to the human cell lines BJAB (CD22+) and Jurkat (CD22−). Both cell lines were incubated for 3 h with various concentrations of the liposomes and analyzed by flow cytometry (Fig. 4A). The anti-CD22 scFv immunoliposomes exclusively bound to the BJAB cell line in a dose-dependent manner. Saturation of immunoliposome binding was achieved at 200 nmol PL/ml. Under these conditions, binding of liposomes to the BJAB cell line is completed within 1 h (Fig. 4B).

3.6. Internalization of anti-CD22 liposomes

To assess whether anti-CD22 scFv immunoliposomes are internalized after binding, flow-cytometric analysis of CD22+ BJAB cells and CD22− control Jurkat cells treated with rhodamine-labeled immunoliposomes was performed. Cells were pre-incubated for 1 h at 4°C with rhodamine-labeled scFv immunoliposomes. After binding of the immunoliposomes, incubation was continued at 37°C to facilitate endocytosis. At various time intervals, cells were treated with protease K to remove cell-surface proteins and bound liposomes from the cells. The remaining rhodamine fluorescence residing in the interior of the cell was detected by flow cytometry (Fig. 5). Results show a time-dependent intracellular accumulation of protease K-resistant rhodamine fluorescence in BJAB cells incubated with anti-CD22 liposomes at 37°C (Fig. 5A) but not in BJAB cells incubated with control anti-IgG liposomes (Fig. 5B). Also, no accumulation of fluorescence was seen in control CD22− Jurkat cells incubated with anti-CD22 liposomes, consistent with their inability to bind these immunoliposomes (Fig. 5C). Internalisation was already apparent 15 min after transfer of cells to 37°C.

Intracellular localisation of rhodamine label was confirmed by confocal laser scan microscopy. BJAB cells were incubated with rhodamine-labeled anti-CD22 liposomes for 1 h at 4°C; cells were then either directly exposed to protease K or incubated at 37°C before digestion with protease K. Cells were fixed to slides and visualized by confocal laser scan microscopy. After incubation for 1 h at 4°C, bright fluorescence is seen associated with the cell membranes (Fig. 6A); this fluorescence could be quantitatively removed by exposing the cells to protease K (Fig. 6B). Incubation of anti-CD22 liposome treated cells at 37°C results in the appearance of bright fluorescent granules in the interior of the cells, in addition to the membrane-associated staining. After incubation of these cells in protease K, only the fluorescent granules inside the cells remained (Fig. 6C,D).

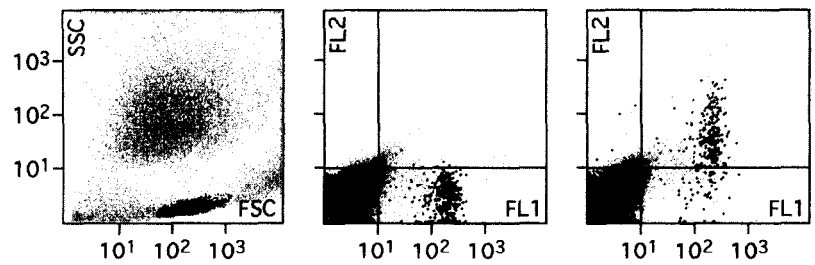

Fig. 3. Binding specificity of anti-CD22 liposomes analysed by flow cytometry. Peripheral blood leukocytes (PBL) were incubated with rhodamine-PE (FL2) labelled immunoliposomes at 4°C for 1 h, washed and double stained with a CD19 FITC (FL1) conjugated monoclonal antibody. Left: forward/sidescatter pattern of PBL. Middle: PBL stained with anti-CD19 FITC (FL1) only. Right: PBL incubated with anti-CD22 liposomes (FL2) followed by staining in anti-CD19 FITC (FL1). Lymphocytes identified by their forward/sidescatter profile are depicted in black.

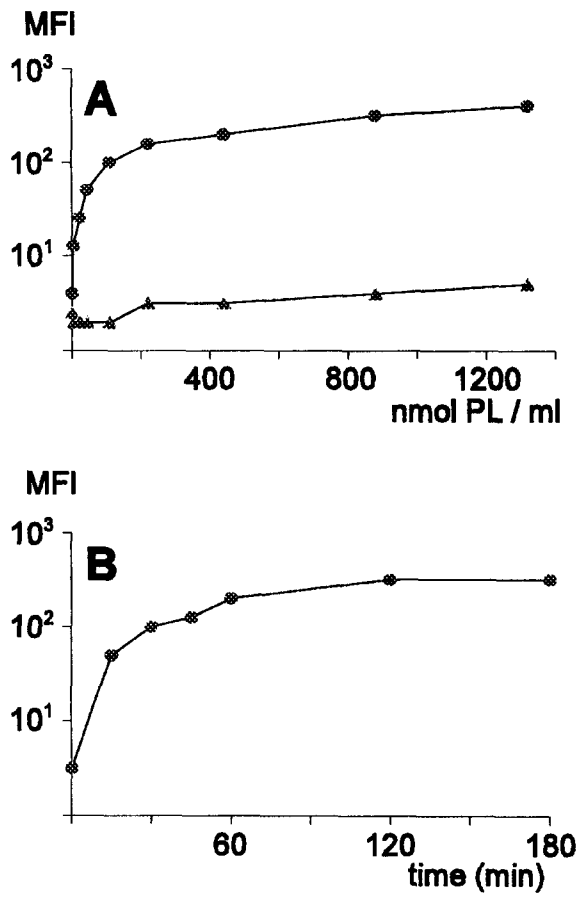

Fig. 4. Flow-cytometric analysis of anti-CD22 scFv immunoliposome binding kinetics. (A) CD22+ BJAB cells (circles) and CD22− Jurkat cells (triangles) incubated for 1 h with different concentrations of rhodamine-PE labelled anti-CD22 liposomes. (B) Anti-CD22 scFv immunoliposomes, at a concentration of 200 nmol PL/ml, incubated with BJAB cells for various periods of time. MFI, mean fluorescence intensity.

4. Discussion

Preparation of conventional immunoliposomes is based on in vitro chemical modification of antibodies for attachment to anchor molecules incorporated into the liposomal surface. Problems encountered with these methods include inactivation of antibodies and low coupling efficiencies. Immunogenicity of murine proteins and rapid removal by cells of the RES pose additional problems associated with the application of immunoliposomes in human therapy [12].

Here, we investigated whether in vivo biosynthetically labeled scFv antibody fragments may represent an improved targeting moiety for incorporation into immunoliposomes. *E. coli* LPP sequences were fused to DNA encoding a human anti-CD22 scFv fragment previously isolated from a synthetic phage display library. A hexahistidyl tag was added to facilitate purification by metal affinity chromatography. Yields of up to 1.6 mg/l purified lipoprotein were repeatedly obtained from detergent-extracted membrane preparations of *E. coli* cultures in shaker flasks. Higher yields of lipoprotein can likely be achieved by high cell density fermentation [13]. Anti-CD22 scFv immunoliposomes were shown to bind rapidly and specifically to CD22+ target cell lines. When incubated with samples of blood leukocytes, the anti-CD22 scFv immunoliposomes exclusively bound to the B-lymphocyte target population and not to other cells in this heterogeneous mixture, indicating that lipid modification and incorporation into liposomes did not alter the scFv fragment's binding specificity.

Antibody-bearing immunoliposomes may be internalized at greatly varying rates, depending on liposome composition, antibody and target receptor [14]. The CD22 receptor is constitutively internalized and degraded in lysosomes, while binding of CD22 antibodies to the receptor enhances CD22 internalization without stimulating lysosomal degradation above the basal constitutive rate [15]. Flow-cytometric and confocal laser microscope analysis demonstrated rapid internalization of anti-CD22 scFv immunoliposomes. The marker fluorochrome concentrated in vesicles located at one side of the cells between the lobes of the nucleus, indicative of an active uptake of immunoliposomes via the endocytotic pathway.

Immunoliposomes represent a versatile class of in vivo delivery systems that may be used in a large variety of applications [16]. Recent advances in liposome technology permit the rational design of vesicles with desired pharmacokinetic properties for the selective delivery of drug, genes and bioactive substances to sub-cellular compartments. We show that in vivo lipid-tagged scFv antibodies from phage display libraries represent promising molecules for the construction of immunoliposomes for cellular targeting. We anticipate that the small size of these molecules, the absence of an immunoglobulin constant region and chemical coupling reagents in these human antibody fragments will reduce their immunogenicity. The availability of large libraries of human scFv fragments and the power of phage display and selection techniques will further facilitate the construction of immunoliposomes with tailor-made properties.

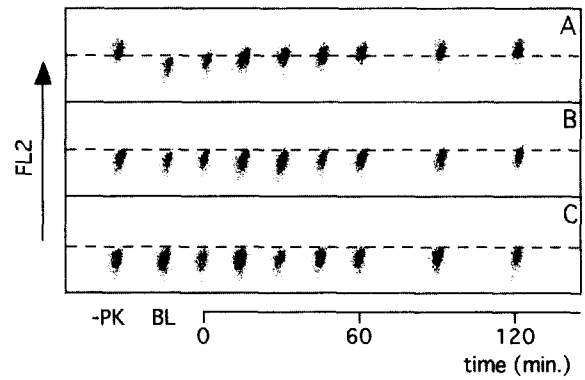

Fig. 5. Internalization of rhodamine-PE labeled anti-CD22 scFv immunoliposomes. Cells were incubated for 1 h at 4°C with immunoliposomes to allow binding. After binding, incubation was continued at 37°C to facilitate endocytosis. At various time intervals samples of cells were treated with protease K and remaining intracellular rhodamine fluorescence (FL2) detected by flow cytometry. Each of the collections of dots corresponds to a separate flow-cytometry profile obtained at the time indicated on the *x*-axis. (A) BJAB cells incubated with anti-CD22 scFv immunoliposomes; (B) BJAB cells incubated with control anti-IgG immunoliposomes; (C) Jurkat cells incubated with anti-CD22 scFv immunoliposomes; BL, blank cells, not incubated with liposomes or protease K; −PK, cells incubated for 1 h at 4°C with immunoliposomes but not treated with protease K.

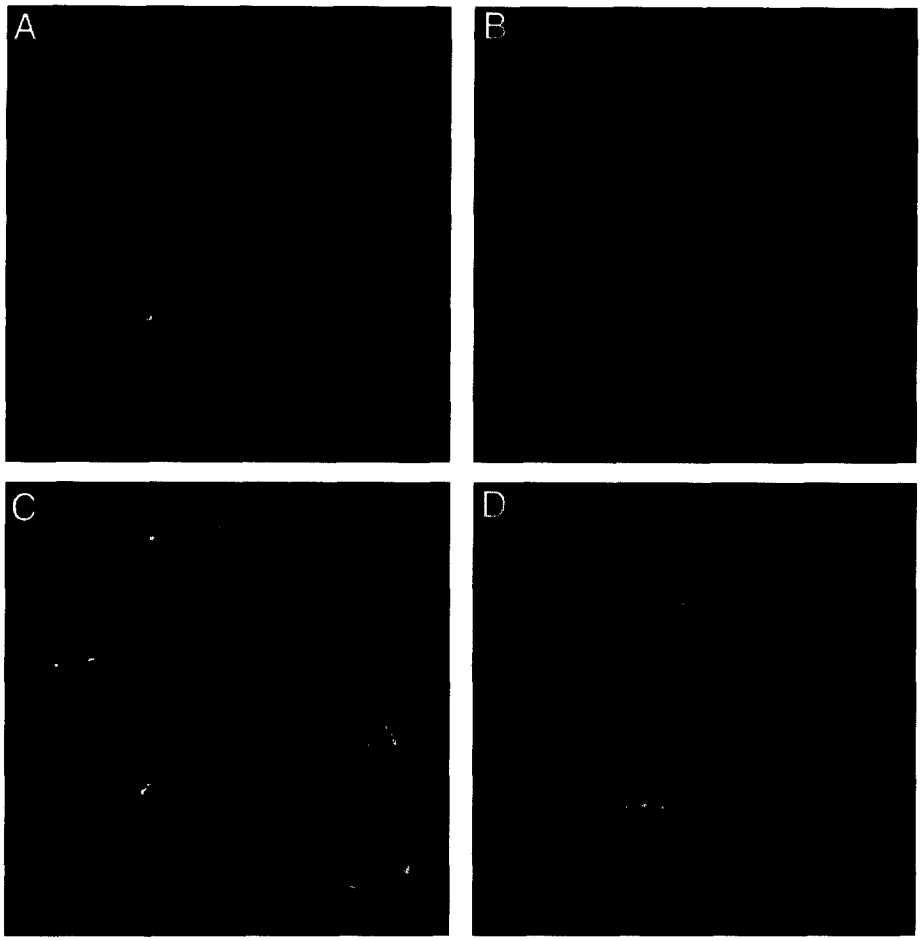

Fig. 6. Internalization of rhodamine-PE labeled anti-CD22 liposomes monitored by confocal laser scan microscopy. CD22+ BJAB cells were incubated with anti-CD22 scFv immunoliposomes at 4°C (A). Cells were then directly exposed to protease K digestion to remove cell-surface proteins and bound liposomes (B), or incubated at 37°C for 1 h (C) before incubation with protease K (D).

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