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Running Head: Cell targeting using scFvs anchored on VLPs via GPI anchor

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1 **ABSTRACT**

2 ***Purpose*** VLPs displaying tumor targeting single-chain variable fragments

3 (VLP-rscFvs) which targets tumor-associated glycoprotein-72 (TAG-72)

4 marker protein have a potential for immunotherapy against colon carcinoma

5 tumors. In this study, scFvs anchored on VLPs using

6 glycosylphosphatidylinositol (GPI) were prepared to target colon carcinoma

7 spheroids in vitro.

8 ***Methods*** VLPs-rscFvs were produced by co-injecting two types of *Bombyx*

9 *mori* nucleopolyhedrovirus (BmNPV) bacmids, encoding RSV-gag and rscFvs

10 cDNA into silkworm larvae. Large unilamellar vesicles (LUVs) of 100 nm in

11 diameter were made using 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)

12 and packaged with Sulforhodamine B (SRB). LUV-SRB was used to associate

13 with VLP-rscFvs assisted by GP64 present on VLP-rscFvs to produce VLP-

14 rscFv associated SRB (VLP-rscFvs-SRB) at pH 7.5.

15 ***Results*** The antigenicity of the purified VLPs-rScFvs was confirmed by

16 enzyme-linked immunosorbent assay (ELISA) using TAG-72 as antigen. LUV-

17 SRB made of DOPC was used to associate with 100 µg of VLP-rscFvs to

18 produce VLP-rscFv-SRB. Specific delivery and penetration of SRB up to 100

19 µm into the spheroids shows the potential of the new model.

20 ***Conclusions*** The current study demonstrated the display, expression and

21 purification of VLP-rscFvs efficiently. As a test model VLP-rscFv-SRB were

22 prepared which can be used for immunotherapy. rscFvs provide the specificity

23 needed to target tumors and VLPs serve as carrier transporting the dye to

24 target.

25 **KEYWORDs** single-chain variable fragment · virus-like particle · silkworm

26 expression system · tumor-associated glycoprotein-72 · large unilamellar

27 vesicle

ABBREVIATIONS

29 CLSM   confocal laser scanning microscope

30 DOPC   1,2-dioleoyl-*sn*-glycero-3-phosphocholine

31 BmNPV   *Bombyx mori* nucleopolyhedrovirus

32 ELISA   enzyme-linked immunosorbent assay

33 gag   group antigen protein

34 GPI   glycosylphosphatidylinositol

35 mAbs   monoclonal antibodies

36 LUVs   large unilamellar vesicles

37 RSV   *Rous sarcoma* virus

38 scFvs   single-chain variable fragments

39 SRB   Sulforhodamine B

40 TAG-72   tumor associated glycoparticle-72

41 VLPs   virus-like particles

42 VLP-rscFvs   VLPs displaying tumor targeting scFVs

43 VLP-rscFv-SRB   VLP-rscFv packaged SRB

44

45 INTRODUCTION

46 Virus like particles (VLPs) are macromolecular structures that are formed using

47 capsid protein from virus which can self-fold to form VLPs and are incapable of

48 infection because they are devoid of genetic material. Group antigen protein (gag)

49 protein from *Rous sarcoma* virus (RSV) species belonging to the family *Retroviridae,*

50 subfamily *Orthoretrovirinae,* genus *Alpharetrovirus* of single stranded RNA virus

51 forms VLPs. gag protein used in the present study is composed of 577 amino acid

52 length with 61 kDa molecular weight, devoid of the protease region (1). It is

53 composed of a membrane-binding domain that directs the gag protein to the plasma

54 membrane via a well-known lipid raft pathway conserved in retrovirus family (2, 3).

55 The interaction domain promotes gag-gag multimerization important for particle

56 assembly to form the VLPs of approximately 80–100 nm and the late assembly

57 domain later facilitates the pinching of viral particles enveloped in a lipid layer from

58 the plasma membrane assisted by proteases (2, 4). Lipid layer can be used to display

59 membrane bound proteins on VLPs surface using glycosylphosphatidylinositol (GPI)

60 anchor domain of proteins (5). The GPI moieties are widely spread in living

61 organisms, playing significant role in providing varying biological functions (6). GPI-

62 anchoring of the proteins takes place in the endoplasmic reticulum membrane at

63 specific site recognized by enzymes enabling the protein to hitch a ride on lipid rafts

64 and reach plasma membrane undergoing the post-translational changes (7, 8). A

65 simple but efficient process, the C-termini of protein covalently attached to GPI via

66 fatty acid chain is stably associated with the membrane. Use of GPI anchored proteins

67 is well-known for detection as well as for targeting disease-causing factors for therapy

68 (9–11). GPI anchored antibodies on VLPs have not yet been studied but antibodies

69 fused with GPI anchored in lipid membranes has been shown to be useful and

70 effective against targets such as HIV-1 envelope spikes for neutralization (12).

71   Immunotherapy has been revolutionized by the successful screening and

72 development of numbers of monoclonal antibodies (mAbs) against specific markers

73 on cancer as a tool for therapy (13, 14). CC49 is a clinically validated antibody to

74 target a tumor-associated glycoprotein-72 (TAG-72) a well-known marker in colon

75 carcinoma (15). mAbs coupled ==with or without== toxins or chemical drugs have been

76 marketed under different names like anatumomab, mafenatox and minretumomab as

77 chemo-therapy treatment an alternative to surgical treatment method (16). mAbs

78 conjugated to drugs or by themself when administered in patients to target tumors

79 have less penetration and only 20% of the administered dose interacts with the tumors

80 (17). mAbs also show clinical bottleneck of aggregation in kidney and other organs

81 involved in removal of toxic materials due to extended biological half-life (2–3 weeks

82 depending on class of antibody) in blood circulatory system (13, 17). Alternatively,

83 single-chain variable fragment (scFvs) linked by a short linker has shown high

84 specificity and relatively low biological half-life (less than 2 h) allowing it to be used

85 as a radio labeled molecule for imaging and diagnosing tumors in patients. Shorter

86 retention time is also one of the main downfalls as the duration its available in the

87 circulatory system is not enough for drug delivery. Different drug delivery systems

88 using scFvs conjugated to drugs or fused with other proteins to be activated on site of

89 target, increase its retention time and enhance the delivery of drug to tumor (15, 18).

90 The biggest disadvantage of such an approach is that modifying the protein by

91 chemical process to tag the scFvs with drugs or enzyme fused with scFvs causes

92 improper folding and loss of scFvs function (19). Hence here a novel approach of

93   displaying recombinant scFvs (rscFvs) anchored with GPI embedded in the lipid layer

94   of VLPs is studied.

95   The silkworm expression system uses silkworm larvae to express and purify

96   proteins efficiently at milligrams level, using *Bombyx mori* nucleopolyhedrovirus

97   (BmNPV), which belongs to the double-stranded DNA virus family *Baculoviridae* (1).

98   BmNPV infects silkworms using spike proteins on baculovirus envelope, like

99   glycoprotein 64 (GP64) a type I membrane protein to associate and fuse with cell

100   membrane to infect silkworms (20–22). GP64 is further classified as type III

101   membrane fusion protein class depending upon its fusion property of initiating fusion

102   independently without any help by forming large fusion pores rapidly. Association of

103   GP64 with membranes takes place first leading to fusion of GP64 to lipid membrane

104   driven by protonation of Histidine amino acids (pK_a 6.0) (23, 24). Previously, it has

105   been reported that RSV gag VLPs expressed and purified using baculovirus

106   expression system have GP64 peppered on its surface (25). Here the use of functional

107   GP64 on VLPs and their association with large unilamellar vesicles (LUVs)

108   membrane in developing a new drug delivery system is studied. The focus is on

109   developing VLPs-rscFvs as a model drug delivery system associated with hydrophilic

110   fluorescent dyes by LUVs. LUVs only packaged with either hydrophilic or

111   hydrophobic fluorescent dyes are well known for studying drug delivery to 3D tumors

112   spheroid model (26–28). The ex vivo 3D spheroid models offer a platform to study

113   and gain insight into drug delivery properties of rapidly evolving new drug delivery

114   tools. The major drawback of using LUVs alone packaged with drugs as a tool for

115   therapy is the non-specificity and the toxicity resulting due to accumulation in

116   detoxification related organs and tissue in humans. LUVs with various marker

117   proteins or baculovirus fused with LUVs have been studied as a drug delivery tool (24,

118 29). Such an approach has either high toxicity or due to use of large baculovirus

119 which carries genetic material causes health and infection concerns and its use is

120 curtailed. Hence the current study focus is on developing a new model system devoid

121 of any genetic material with high target specificity provided by rscFvs anchored on

122 VLPs by GPI carrying a test fluorescent dye.

123 In the current study, feasibility of using VLPs a macromolecular structure

124 displaying rscFvs targeting cancer cells and spheroids made from the same cells has

125 been tested. The current approach uses GPI to anchor scFv on VLPs without putting

126 any protein folding constraints on either scFv-GPI or gag-gag multimerization for

127 VLPs formation. In addition, LUVs packaged with sulforhodamine B (SRB) a water-

128 soluble fluorescent dyes to associate with VLPs displaying rscFv and GP64 as a

129 model to test an effective drug delivery. SRB a water soluble negatively charged

130 aminoxanthine dye is used for cell based cytotoxicity assay in various cancer cell

131 lines with better accuracy than other known assay (30). Utilizing GP64 is important

132 for large-scale application because VLPs displaying rscFvs can be made with ease by

133 the silkworm expression system and the VLPs can serve as carrier of drugs or dyes to

134 give the extra merit needed in rapidly evolving drug delivery systems targeting

135 tumors.

MATERIALS AND METHODS

136

137 Cell lines and Media

138 LS174T human colon adenocarcinoma cell line (ATCC CL-188) was obtained from

139 ATCC (Manassas, VA, USA) and HEK293 (RCB1637) was obtained from Riken Bio

140 Resource Center (Tsukuba-shi, Ibaraki, Japan). LS174T presenting a tumor associated

141 glycoprotein-72 (TAG-72) on cell surface, was cultured in 60 mm culture plates (TPP,

142   Trasadingen, Switzerland) with MEM-eagle medium (Sigma-Aldrich, St. Louis, MO,

143   USA) containing 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA),

144   supplemented with 1% (v/v) antibiotic solution containing penicillin, streptomycin,

145   fungizone (Sigma-Aldrich) and incubated at 37°C in 5% CO_2 incubator (MCO-175

146   Sanyo, Osaka, Japan). HEK293 were cultured in 60 mm culture plates with

147   MEM/EBSS (HyClone Laboratories Inc., Utah, USA) containing 2 mM L-glutamine,

148   1% non-essential amino acid (Invitrogen), 10% fetal bovine serum, supplemented

149   with 1% (v/v) antibiotic solution containing penicillin, streptomycin, fungizone and

150   incubated at 37°C in 5% CO_2 incubator. Both the cell lines were grown till confluence

151   and splitting was done in 1 : 5 ratio once every week by trypsinisation using TrypLE

152   Express (Life Technologies Japan LTD., Minato-Ku, Tokyo, Japan) for 15 minutes at

153   37°C in 5% CO_2 incubator.

154   PCR Amplification and Bacmid Preparation

155   Using PredGPI software the GPI anchor position and the nucleotide sequence was

156   confirmed as previously reported for VLPs displaying rNcSRS2 (31) and the same

157   GPI anchor is used in current work. The plasmid carrying CC49 gene was kindly

158   provided by Professor Hiroshi Ueda (Tokyo Institute of Technology, Japan). The scFv

159   cDNA was cloned by PCR into pENTR to make pENTR/scFv-DDDDK. The GPI

160   anchor cDNA was inserted in frame by PCR using pENTR/scFv-DDDDK plasmid as

161   template using forward primer 5'-

162   caccatg**aagatactccttgctattgcattaatgttgtcaacagtaatgtgggtgtcaacagactacaag**<u>gatgacgat</u>

163   <u>gacaag</u>-3' and reverse primer 5'-

164   tta<u>tcagtacgcaaagattgccgttgcagtcagtgacgcagcggatagtgccacgtacgaaggcaactcgtcacatgcatc</u>

165   <u>t</u>ccggatcccccccccccgtttaa-3' respectively. The forward primer contained 63 base pair

166 Bombyxin signal (bold) sequence followed by DDDDK affinity tag nucleotide

167 sequence (dashed line). The reverse primer contained the 81 base pair of putative GPI

168 signal (underlined) motif of NcSRS2. PCR products were inserted into the entry

169 vector, pENTR/D-TOPO (Invitrogen) to give pENTR/scFv-GPI. The PCR fragment

170 inserted into pENTR/D-TOPO was confirmed by dideoxynucleotide chain

171 terminating sequence (32) using Thermo Sequenase Cycle Sequencing kit (USB Co.,

172 Cleveland, Ohio, USA). pENTR/scFv-GPI was used to transfer scFv-GPI to pDEST8

173 by LR reaction according to the protocol to make pDEST8/scFv-GPI, which was used

174 to transform to *E. coli* BmDH10bac competent cells to make recombinant bacmids

175 (33). White colonies of recombinant bacmid carrying scFv-GPI gene were isolated

176 and resulting bacmid was designated as BmNPV bacmid/VLP-rscFvs.

177   Bacmid/RSV-gag-577 from previously reported work is used (1) and the

178 BmNPV bacmids were isolated and resuspended in phosphate buffered saline

179 containing 80 mM di-sodium hydrogen orthophosphate anhydrous, 19 mM sodium di-

180 hydrogen orthophosphate anhydrous, and 100 mM sodium chloride (PBS, pH 7.5) for

181 injection.

182 Silkworm Larvae Rearing, Feeding and Injection

183 Fifth instars larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were reared on an

184 artificial diet Silkmate S2 (Nihon Nosan Kogyo, Yokohama, Japan) in a 65%

185 humidity chamber (MLR-351H, Sanyo, Tokyo, Japan) at 27°C.

186   Each silkworm was injected with 40 μL recombinant bacmid DNA solutions

187 containing 10 μg of BmNPV-gag577 and BmNPV-scFv-GPI bacmids, respectively, in

188 10% (v/v) DMREI-C reagent (Invitrogen) in PBS using 1 mL syringe. Post injection

189 7th day the silkworm larval hemolymph was harvested in tubes (Falcon, Lincoln Park,

190 NJ, USA) containing 2 mM phenyl thiourea and complete EDTA-free protease

191 inhibitor cocktail to inhibit the hemolymph melanization and protein degradation by

192 proteases. These samples were aliquoted into eppendorf tubes and stored at -80°C.

Purification of VLPs Displaying scFvs-GPI

193

194 VLP-rscFvs containing hemolymph collected from silkworm larvae were centrifuged

195 at 1,000 g in a Heraeus Primo R Sorvall Biofuge (Thermo Scientific, Yokohama,

196 Japan) for 3 min using Heraeus 7591 swing bucket rotor to remove debris. The clear

197 hemolymph was dialyzed with cellulose ester dialysis membrane (Spectrum

198 Laboratories Inc., California, USA) having 300,000 molecular weight cut off in 2 L of

199 PBS (pH 7.5) for overnight at 4°C. The dialyzed hemolymph was centrifuged at

200 14,010 g using micro refrigerated centrifuge (Model 3700, Kubota, Tokyo, Japan) for

201 10 min at 4°C to remove any aggregates and the supernatant was filter-sterilized with

202 5 μm filter membrane (Merck-Millipore, MA, USA). The protein sample was added

203 to PBS pre-equilibrated 5 mL of DDDDK-agarose gel (Medical and Biological

204 Laboratories Co., Ltd., Nagoya, Japan) in batch mode and purification performed as

205 per the kit protocol. Elution was carried out using 0.1 mg/mL DDDDK peptide

206 (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) in 500 μL aliquots. The

207 protein concentrations were measured using standard BCA protein estimation kit

208 (Pierce BCA Assay kit, IL, USA).

Western-blot Analysis

209

210 To detect the expression of VLPs and rscFvs, larval hemolymph from silkworm

211 larvae were collected. Samples were subjected to 10% (w/v) SDS-PAGE using the

212 mini-protean II system (Bio-Rad, Hercules, California, USA). After SDS-PAGE,

213 proteins were blotted on to a PVDF membrane using the Mini Trans-Blot

214 Electrophoretic Transfer cell (Bio-Rad) at 15 V for 1 h. The membranes were probed

215 with 5,000 and 2,000 fold diluted mouse anti-DDDDK primary antibodies (Wako

216 Pure Chem. Ind. Ltd., Osaka, Japan) for rscFvs and rabbit anti-RSV-gag primary

217 antibody for gag-577 (1), respectively, in Tris-buffered saline with 0.1% (v/v) Tween-

218 20 (TBS-T) (Wako) for 1 h at room temperature with mild shaking. Secondary

219 antibodies conjugated with horseradish peroxidase (HRP) were goat anti-mouse IgG

220 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for rscFvs and goat anti-rabbit

221 IgG (Santa Cruz Biotechnology) for gag-577, respectively. The secondary antibodies

222 were incubated for 1 h at room temperature and specific bands for rscFvs and gag-577

223 proteins were detected using an Immobilon western blotting reagent pack (Millipore

224 Corporation, Billerica, Massachusetts, USA). The rscFvs and gag-577 proteins bands

225 were analyzed using a Fluor-S/MAX multi-imager (Bio-Rad).

226 Confirmation of the Antigen Specificity of rscFvs Displayed on VLPs by ELISA

227 Human TAG-72 at 20 U per well (Sigma-Aldrich) in 100 μL volume was

228 immobilized in an immuno plate (Thermo Scientific, West Palm Beach, FL, USA),

229 overnight at 4°C in triplicates. The plates were blocked with 100 μL/well Ez-block

230 Chemi (ATTO Co., Tokyo, Japan) for 1 h at room temperature and washed thrice with

231 200 μL/well PBS (pH 7.5). Five micrograms of VLP-rscFvs and VLPs were added to

232 each well, respectively and incubated for 2 h at room temperature. After incubation,

233 the plates were washed three times with 200 μL/well PBS (pH 7.5). The plates were

234 incubated with 100 μL/well mouse anti-DDDDK antibody 2,000 fold diluted in PBS-

235 T (Tween-20 0.1% (v/v)) and incubated for 1 h at room temperature. After incubation

236 the plates were washed thrice with 200 μL/well PBS-T pH 7.5 and incubated with

237 goat anti-mouse IgG conjugated with HRP 4,000 fold diluted in PBS-T for 1 h at

238 room temperature. After incubation the plates were washed thrice with 200 μL/well

239 PBS-T (pH 7.5) and detection was carried out using 3,3'-,5,5'-tetramethylbenzidine

240 (TMBZ) (Dojindo, Kumamoto, Japan) solutions by observing the absorbance at 450

241 nm by Plate reader (Bio-Rad) (5).

242 Phospholipase C Treatment of VLP-scFv-GPI

243 Each well of immuno plate was immobilized with 5 μg of VLP-rscFvs and VLPs,

244 respectively for overnight at 4°C in triplicates. The plates were blocked and treated

245 with primary and secondary antibodies similar to ELISA method. After incubation the

246 plates were washed three times with 200 μL/well PBS-T (pH 7.5) and presence of the

247 GPI anchor from the lipid layer was confirmed by digestion with 0.1 U of

248 phosphatidyl-inositol specific Phospholipase C (PI-PLC) (Sigma-Aldrich) in 200

249 μL/well PBS (pH 7.5) for 2 h at 27°C. After incubation the plates were washed and

250 scFv was detected similar to ELISA method.

251 Hemolysis Assay

252 Rabbit erythrocytes (Nihon BioTest Research, Tokyo, Japan) were prepared and 2.5%

253 (v/v) were seeded per 96-well plate in triplicates (34). Purified VLP-rscFvs and

254 Bovine serum albumin (100 μg/mL) as a negative control were added and allowed to

255 absorb for 10 min on ice. Then, the mixture was incubated for 30 min at 37°C. Extent

256 of hemolysis was determined spectrophotometrically at 540 nm in a plate reader (Bio-

257 Rad).

258 Liposome Preparation and Sulforhodamine B Packaging

259   Multilamellar vesicles (MLVs) were prepared using 10 mM 1,2-dioleoyl-*sn*-glycero-

260   3-phosphocholine (DOPC) (Avanti Polar Lipids, Alabaster, Alabama, USA) dissolved

261   in chloroform (Wako) in 5 mL glass vial with a cap. DOPC was evaporated under

262   flowing nitrogen gas, films formed at the bottom of the vial and were kept in a

263   desiccator in vacuum overnight for complete drying of chloroform. Films were then

264   hydrated by adding PBS (pH 7.5) containing 1 mM sulforhodamine 101 acid chloride

265   (SRB) (Dojindo, Kumamoto, Japan) at room temperature by vortexing five times for

266   20 seconds. SRB was packaged into MLVs by freeze thawing method in a three-step

267   cycle of 20 min. MLVs were extruded using Avanti Mini-extruder (Avanti Polar

268   Lipids) with a 19 mm diameter polycarbonate 100 nm pore size membrane (Avestin,

269   Ontario, Canada) 20 times to produce LUVs packaged with SRB (LUV-SRB). LUV-

270   SRB mixture containing LUVs and LUVs-SRB were resolved on pre-equilibrated

271   sephadex G75 (7 cm × 3 cm dimensions) column (Terumo, Tokyo, Japan). The

272   purified LUV-SRB suspension was collected from the void volume fraction and

273   concentration of the lipid and SRB were estimated (35). Lipid estimation was done by

274   Fiske-Subbarrow colorimetric method using LUV-SRB prepared in HEPES buffer pH

275   7.5 under conditions similar as described above. The SRB excitation and emission

276   measurements were taken at 515 and 604 nm, respectively by a 96 well

277   spectrophotometer (Tecan Japan Co., Ltd., Kawasaki, Japan).

278   One hundred micrograms of VLP-rscFvs in 500 μL of PBS (pH7.5) buffer

279   were mixed with 500 μL of LUV-SRB containing 28.8 μM DOPC and 1 mM SRB.

280   The mixture VLP-rscFv-LUV-SRB (VLP-rscFv-SRB) was incubated for 1 h at 27°C

281   for association to be complete. Small aliquot from VLP-rscFv-SRB sample was used

282   for SRB concentration measurement.

283   Dynamic Light Scanning (DLS) Measurement

284 One hundred micrograms of purified VLP-rscFvs and VLP-rscFv-SRB were extruded

285 using Avanti Mini-extruder (Avanti Polar Lipids) with a 19 mm diameter

286 polycarbonate 200 nm pore size membrane (Whatman Japan Ltd., Tokyo, Japan)

287 three times to get uniformly dispersed sample. The samples were loaded in disposable

288 cuvettes (DTS-1061) for measurement of size with the Zetasizer Nano series

289 (Malvern, Worcestershire, United Kingdom).

290 Transmission Electron Microscopy (TEM) of VLP-scFvs-GPI and its

291 Packaging

292 The purified VLPs-rscFvs, LUV-SRB and VLP-rscFv-SRB samples respectively were

293 spotted on carbon grids (Okenshoji, Tokyo, Japan) and dried at room temperature.

294 Negative staining was performed using 2% (v/v) phosphotungstic acid (Wako) as

295 described previously (5). For immunoelectron microscopy, VLPs-rscFvs and VLP-

296 rscFv-SRB samples were loaded on the grids in similar fashion and the grids were

297 blocked using 4% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h and

298 washed with PBS (pH 7.5). The grids were incubated in PBS containing mouse anti-

299 DDDDK at 1:100 dilutions for 2 h, and washed with PBS. Subsequently, the grids

300 were incubated in PBS containing 1:200 diluted goat anti-mouse IgG conjugated with

301 10 nm gold particles (BB International, Cardiff, UK) for 2 h, and washed with PBS.

302 Negative staining was performed as mentioned above and samples were observed at

303 50,000 × magnification (JEM 2100F-TEM, JEOL Ltd., Akishima, Tokyo, Japan)

304 operating at 200 kV. The high-resolution TEM images were transformed using fast

305 Fourier transformation function available with the instrument.

306 Confirming Specificity of VLP-rscFvs

307 LS174T and HEK293 cell lines were cultured for 3 days and around 10^4 cells were

308 seeded on glass slides (2.6 cm × 7.6 cm) (Matsunami Glass Ind., LTD., Osaka, Japan)

309 and incubated overnight under growing conditions. The cells were washed

310 respectively with fresh medium and incubated with 3 μg of VLP-rscFvs for 3 h. The

311 cells respectively were treated with 500 fold diluted BODIPY FL-C5-ceramide

312 conjugated to BSA (Invitrogen) and mouse monoclonal anti-DDDDK-tag-Alexa

313 Fluor 594 (MBL Co. LTD., Nagoya, Aichi, Japan) respectively and incubated for 1 h

314 at 37°C. The cells were then washed with fresh growth medium and incubated with

315 1,000 fold diluted 4'-6-Diamidino-2-2-Phenylindole, Hydrochloride (DAPI) solution

316 for 1 h at 37°C. The cells were washed once respectively, fixed using 2% (v/v)

317 formaldehyde and viewed under confocal laser scanning microscope (CLSM) (LSM

318 700, Carl Zeiss, Oberkochen, Germany) with Plan-apochromat 40 × (2.5 zoom factor)

319 oil lens respectively. Zen LE software available on Carl Zeiss website was used for

320 image analysis.

321   Similar amount of VLP-rscFvs as used above was also fixed on glass slides

322 and treated with 500 fold diluted mouse monoclonal anti-DDDDK-tag-Alexa Fluor

323 594 and incubated for 1 h at 37°C. VLP-rscFvs samples were not stained with DAPI

324 as there was no nucleus present. The samples were fixed as mentioned above and

325 observed under CLSM with Plan-apochromat 100 × (1 zoom factor) oil lens. Zen LE

326 software available on Carl Zeiss website was used for image analysis.

327 Delivery of VLP-rscFv-SRB

328 LS174T cells as mentioned above were cultured and seeded on the slides. The cells

329 were washed with fresh medium and incubated with 3 μg of VLP-rscFv-SRB and 100

330 μL of LUV-SRB as a negative control for 3 h. The cells were washed and treated with

331   500 fold diluted mouse monoclonal anti-DDDDK and incubated for 1 h at 37°C. The

332   cells were washed and treated with 1000 fold diluted FITC conjugated goat anti-

333   mouse IgG (Jackson ImmunoResearch lab., Baltimore, Maryland, USA) and

334   incubated for 1 h at 37°C. The cells were washed once respectively, fixed using 2%

335   (v/v) formaldehyde and viewed under CLSM with Plan-apochromat 100 × oil lens

336   and 20 ×, respectively. Zen LE software available on Carl Zeiss website was used for

337   image analysis.

338   Large Spheroids Preparation and VLP-rscFv-SRB Delivery

339   Large spheroids mold of 0.8 mm × 0.8 mm (diameter × depth) with 5 × 7 array using

340   3D Petri dish (MicroTissues Inc., Rhodes Island, USA) were used to produce 500 μm

341   diameter spheroids as per kit protocol. LS174T cells resuspended in 200 μL growth

342   medium (1.4×10^5 cells) were applied to equilibrated spheroid molds kept in 60 mm

343   cell view cell culture plates (Greiner Bio-one GmbH, Maybachstr. 2, Germany) and

344   incubated at 37°C in CO_2 incubator for 30 minutes resulting in large spheroid

345   formation, confirmed with 2 × lens of Olympus SZX14 microscope (Olympus, Tokyo,

346   Japan).

347   One hundred micrograms of VLP-rscFv-SRB mixture were added to spheroids

348   and incubated at 37°C in CO_2 incubator for 3 h. The spheroids were then washed with

349   growth medium and treated with 250 fold diluted mouse monoclonal anti-DDDDK in

350   500 μL growth media and incubated at 37°C in CO_2 incubator for 1 h. The spheroids

351   were washed with fresh growth medium and treated with 500 fold diluted goat anti-

352   mouse IgG (H+L) conjugated with FITC (Jackson ImmunoResearch lab.) in 500 μL

353   growth medium and incubated at 37°C in CO_2 incubator for 1 h. Finally the cells

354   were washed with growth medium and observed under live condition using 10 ×

355   apochromat lens of CLSM. Z-stacking was performed for 338 μm in-depth of

356   spheroid with each cross-sectional scanned layer of 1 μm. All the Z-stacked images

357   collected were rendered to prepare the 3D model using Zen LE 2011 version software

358   available on Carl Zeiss website.

359   **RESULTS**

360   Expression and Purification of rscFvs and gag Proteins

361   Expression of rscFvs (~32 kDa) and gag protein was confirmed in purified samples

362   collected from silkworm larvae hemolymph co-injected with both BmNPV/RSV-gag-

363   577 and BmNPV/rscFvs bacmids. The western blot data for gag confirmed with

364   molecular weight as reported earlier (Fig. 1A) (4). The western blot data for rscFvs

365   shows another 35 kDa band (Fig. 1B), the apparent increase by about 3 kDa results

366   due to the cells inability to remove the Bombyxin signal sequence or the GPI anchor

367   whose processing might be one of the reasons. Approximately 1.5 milligrams of

368   purified VLP-rscFvs was isolated from 30 mL of silkworm larvae, and shows the

369   presence of the rscFvs and gag bands in CBB stained SDS-PAGE gel (Fig. 1C).

370   Confirmation of Antigenicity and GPI Anchorage

371   The rscFvs specificity to bind to TAG-72 the marker on colon carcinoma is an

372   important property. The antigen specificity of rscFvs was confirmed by ELISA using

373   TAG-72 antigen. Compared to negative control without rscFvs, specificity of VLP-

374   rscFvs for TAG-72 was 22 fold higher (Fig. 2A).

375   The GPI anchorage of rscFvs was investigated using PI-PLC treatment. PI-

376   PLC treated wells compared to untreated wells showed around 40% decrease in signal

377   (Fig. 2B) indicating presence of GPI anchored scFv. VLPs only as negative control

378   show no remarkable change.

379   **TEM and ImmunoTEM Analysis of VLP-scFvs and VLP-rscFv-SRB**

380   TEM images of purified VLPs-rscFvs show a distinctive bilayer that is usually

381   present on enveloped VLPs with a diameter of 100 nm (Fig. 3A). The immuno-TEM

382   images of VLP-rscFvs show presence of rscFvs particles on the surface of VLPs (Fig.

383   3B). TEM images of purified LUVs packaged with SRB shows distinctive smooth

384   shaped LUVs of approximately100 nm in diameter (Fig. 3C). TEM images of VLP-

385   rscFv-SRB associated with LUVs-SRB shows the lipid bilayer. The average size of

386   VLP-rscFv-SRB from TEM images is around 120–150 nm in diameter (Fig. 3D).

387   Immuno-TEM of VLP-rscFv-SRB shows presence of rscFvs particles on the surface

388   of VLP-rscFv-SRB (Fig. 3E). VLP-rscFv-SRB TEM images show the association

389   between VLP-rScFvs and LUV-SRB as shown by the arrows (Fig. 3F). Both VLP-

390   rscFv-SRB and LUV-SRB have a high contrast but the distinctive bilayer of VLPs

391   easily distinguishes the nanostructures and suggests that VLP-rscFvs can bind

392   strongly to LUV-SRB at pH 7.5. The samples were passed through 200 nm

393   nucleopore membrane to avoid aggregation among VLP-rscFvs before measuring size

394   by DLS for quantitative analysis of size change. The peak of the number distribution

395   for the diameter of LUV-SRB, VLP-rscFvs and VLP-rscFv-SRB from DLS data was

396   around 68, 51, and 79 nm, respectively (Fig. 3G). The shift in diameter for VLP-

397   rScFvs and VLP-rscFv-SRB from DLS was of 28 nm. The increase in diameter of

398   VLP-rscFv-SRB might be due to association or fusion of LUV-SRB with VLP-rscFvs

399   suggesting that VLP-rscFvs can bind strongly to LUV-SRB at pH 7.5. LUV-SRB

400   association with VLP-rscFvs results due to presence of GP64 on VLP-rscFvs surface.

401   The result of hemolysis indicates that VLP-rscFvs-SRB associated with RBCs

402   strongly to induce hemolysis at pH 7.5 (Fig. 4), suggesting that VLP-rscFvs can bind

403   strongly LUV-SRB. The negative control using BSA showed zero percent hemolysis

404   (Data not shown).

405   Localization of VLP-rScFvs and VLP-rscFv-SRB

406   Purified VLP-rscFvs were viewed under CLSM, and 0.1–0.2 μm big spots were

407   observed after treatment with Alexa Fluor 594 conjugated anti-DDDDK (Fig. 5A–C),

408   supporting that the presence of rscFvs in purified VLP-rscFvs as shown in the

409   schematic representation (Fig. 5D). The purified VLP-rscFvs show specificity to the

410   TAG-72 antigen present on LS174T cells surface (Fig. 6A–D). The rscFvs presence

411   and its localization were confirmed in LS174T cells (Fig. 6C and D). As a negative

412   control, LS174T cells were treated with VLPs without rscFvs and they show no

413   fluorescence for the affinity tag of scFv (Fig. 6E–H). Purified VLP-rscFVs was added

414   to HEK293 cells to further confirm the specific binding of the VLP-rscFvs

415   irrespective of any influence from lipid layer around VLPs, shows only plasma

416   membrane localizing dye and no fluorescence for rscFvs (Fig. 6I–L). Thus VLP-rscFv

417   can bind to LS174T cells specifically via scFv for TAG-72 antigen.

418   Association of VLP-rscFvs with SRB and Targeting the Cells and Spheroids

419   LS174T cells with and without VLP-rscFvs-SRB treatment confirmed the delivery of

420   SRB to cells (Fig. 7A–F). Approximately 3 μg of VLP-rscFvs-SRB treated cells show

421   FITC fluorescence for the affinity tag of rscFvs and SRB, respectively (Fig. 7A and

422   B). In negative control cells treated with LUV-SRB or 1mM SRB only showed

423   insignificant fluorescence of FITC and SRB (Fig. 7D-E and Supplementary Fig. 3).

424   VLP-rscFvs-SRB binding to cancer cells is facilitated by rscFvs specificity for TAG-

425   72, leading to the delivery of SRB the test model dye to cell (Supplementary Fig. 1).

426   Large spheroids of 500 micrometers in diameter were produced using 3D

427   agarose scaffold (5 × 7 arrays) (Supplementary Fig. 2). The spheroids were spherical

428   and there was no aggregation of cells on the scaffold body other than the intended

429   area. Approximately 2.8 μg of VLP-rscFv-SRB per spheroid was incubated and

430   fluorescence of SRB and FITC channels was observed as shown schematically from

431   bottom (Fig. 8A). All the images for Z-stacking of 1 μm thickness were taken from

432   the bottom to top. The rendered images showed SRB at a depth of 100–150 μm from

433   the bottom of spheroid (Fig. 8B). FITC denoting rScFvs was observed at a depth of

434   about 150–250 μm from the bottom of the spheroid (Fig. 8C). The depth indicator

435   from the merged picture shows there is overlap between SRB and rScFvs respectively

436   at 100–150μm from the bottom as observed by the yellow color due to mixing of red

437   and green channel colors (Fig. 8D). SRB (Fig. 8B) fluorescence and scFvs signals

438   (Fig. 8C) were overlapped on the surface of the spheroid, indicating that scFv-

439   displaying VLP bound to the cells on the surface of LS174T spheroid and carried

440   SRB to these cells.

441   **DISCUSSION**

442   Many kinds of drug delivery systems targeting tumors for therapy using liposome's

443   and VLPs of different compositions and proteins are being developed for delivering

444   genes or drugs to mammalian cells (16, 36, 37). Here the novel use of VLPs

445   displaying rScFvs with SRB as a model dye was reported to confirm the hypothesis of

446   using VLP-rscFvs as a new drug delivery system. In order to develop the delivery

447   system an expression system using silkworm larvae was established and VLP-rscFvs

448 expression and purification in milligram levels was confirmed. rscFvs expression and

449 its display on the VLPs was facilitated by bombyxin signal sequence from *Bombyx*

450 *mori* added to N-Terminal side of rscFvs (5). The signal sequence facilitates the

451 rscFvs to reach the plasma membrane where VLPs formation takes place and they are

452 secreted into silkworm larval hemolymph. gag protein shows different bands due to

453 protease activity present in silkworms hemolymph as shown before (4, 18, 23). gag

454 (~42 kDa) protein can be confirmed by CBB staining but the other band intensities

455 were low for detection by CBB staining.

456   Many reported approaches use scFvs conjugated to drugs or proteins, but

457 bottleneck of efficient conjugation and the resulting loss of function is a leading cause

458 for less use of scFvs for therapy (17). In the present study, the high specificity of

459 rscFvs displayed on VLPs is directly related with proper folding as there is no loss of

460 function. The current protein expression model expresses two or more proteins

461 independent of each other and the assembly takes place on the plasma membrane

462 independent of each other with no protein folding constraints due to fusion. Thus the

463 assembled proteins retain their native structures and functions. gag protein self

464 assembly is well documented, its well suited for forming VLPs and display of foreign

465 proteins (1, 5). Use of GPI anchor to display protein embedded in VLPs lipid bilayer

466 and retaining the high antigenicity at the same time has been reported previously (5).

467   In the present study, a novel strategy employing GPI anchored scFvs on

468 macromolecular nano-structures is tried. GPI anchoring was confirmed by PI-PLC

469 treatment, an enzyme specifically cleaving the phosphodiester bond anchoring the

470 protein to fatty acid chains in lipid layer of VLPs. The high antigen specificity of

471 rscFvs against TAG-72 showed that the displayed protein is properly folded with no

472 loss in function due to anchoring. VLP-rscFvs and VLP-rscFv-SRB displaying rscFvs

473 was confirmed by TEM and the qualitative analysis of TEM images shows the size

474 increase. To understand the size increase of VLP-rscFv and VLP-rscFv-SRB a

475 quantitative analysis using DLS was done, which showed a spread out in size as the

476 current, expression system produces VLPs of varying size displaying different

477 number of proteins. The exact mechanism involved in the current method to produce

478 VLP-rscFv-SRB is unclear leading to association of LUV-SRB with VLP-rscFv and

479 further work is needed.

480   Small scFvs anchored using GPI on VLPs can serve as an important tool for

481 targeting cancer markers and support a robust delivery system. Here we propose using

482 VLP-rscFv-SRB a composite macromolecular nanostructure with SRB to be delivered

483 to spheroids in vitro as a model. The shape of the VLPs-rscFvs was similar as that of

484 the VLPs (25). During the assembly of the macromolecular nanostructure GP64 is

485 also peppered on top of VLPs due to the use of BmNPV expression system in

486 silkworms as reported before (25). The apparent increase in diameter of VLPs-rscFvs

487 could be attributed to insertion of the rscFvs and GP64 protein molecules together in

488 between the gag monomers when the macromolecular nanostructure assembly takes

489 place. The mechanism and number of proteins inserted per VLPs is not clear and

490 needs to be further investigated. But GP64 is a well-studied fusion protein that forms

491 large pore and due to presence of GP64, VLP-rscFvs with relative ease could be

492 associated with SRB using LUV-SRB at neutral pH. To support this hypothesis GP64

493 function peppered on VLP-rscFvs was confirmed by hemolysis assay. The exact

494 mechanism behind the association using LUV-SRB and VLP-rscFv needs to be

495 further studied. rscFvs presence was confirmed on VLPs before and after fusion with

496 LUVs-SRB. Association of dye did not influence presence of rscFvs opening the

497 prospect of displaying multiple proteins on top of VLPs to target various types of

498 tumors in future.

499   Cancer cell based assay reported here uses the binding specificity of VLP-

500 rscFvs to TAG-72 present on the cancer cells surface. As a negative control HEK293

501 cells were used without any TAG-72 on its surface to show the binding specificity.

502 The binding specificity to TAG-72 on cancer cell plasma membrane was confirmed

503 by co-localization studies using membrane-localizing dye. VLP-rscFv-SRB functional

504 property of binding was also confirmed in a similar manner with cancer cells, which

505 confirmed association of LUV-SRB to VLP-rscFv, does not hinder in the binding

506 specificity of rscFv. Here we propose a hypothesis wherein the rscFvs on VLP-rscFv-

507 SRB bind with TAG-72 present on the cells leading to the delivery of the dye to cells

508 (Supplementary Fig. 1). The presence of both SRB and rscFv co-localization takes

509 place as observed by the merged yellow color resulting due to mixing of red and

510 green color channels of the dye. Based on this hypothesis VLP-rscFv-SRB delivery of

511 SRB to spheroids were confirmed by the co-localization of rscFvs and SRB. The

512 presence of both SRB and rscFvs and its depth in the spheroids based on the rendered

513 image generated using the z-stacked image is about 100–150 μm within 3 h of the

514 incubation period. SRB is well known for cancer cell cytotoxicity assay studies owing

515 to its sensitivities but the primary motive here was to show the delivery of the dye to

516 the spheroids. The endocytosis pathway and the penetration property of the

517 fluorescent probe SRB needs to be further investigated. In the current research it was

518 used as a model for confirming the delivery only.

519 **CONCLUSIONS**

520   This study shows two novel aspects, first is the novel use of GPI anchors successfully

521   displaying rscFvs on top of VLPs. By placing rscFvs specific towards TAG-72

522   displaying on top of the VLPs the biofunctional ability of VLPs as a composite

523   macromolecular nanostructure were obtained to target tumors. In order to test this

524   hypothesis VLP-rscFvs associated with LUV-SRB were used here as a test model to

525   deliver to spheroids, a well-studied ex vivo tumor model. The second novel aspect of

526   this study is the use of GP64 present on VLPs surface due to silkworm expression

527   system for producing VLPs-rscFv-SRB at neutral pH. Thus efficient expression can

528   be done using silkworm expression system and the purified VLP-rscFvs can be

529   associated with dyes in a simple way facilitated by GP64. In future other known drugs

530   for colon carcinoma can be tried using similar principle.

531   ACKNOWLEDGEMENTS

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644

FIGURE LEGENDS

646 **Fig. 1.** Western blotting of purified samples of VLP-rscFvs. gag-577 (A) and

647 rscFvs (B) were detected by western blotting using rabbit anti-gag primary

648 antibody and mouse anti-DDDDK primary antibody, respectively. Secondary

649 antibodies used for detection of gag-577 (A) and rScFvs (B) were goat anti-

650 rabbit IgG and goat anti-mouse IgG, respectively. Lane 1: molecular weight

651 marker; lane 2: 1 µg of VLP-rscFvs samples. (C) CBB stained 10% SDS-

652 PAGE gel of VLP-rscFvs samples. Lane 1: molecular weight marker, lanes 2

653 and 3: 1 µg and 5 µg of VLP-rscFvs samples, respectively. The black and

654 white arrows show the rscFvs and gag (some minor cleaved bands of gag are

655 also observed between 40~61 kDa), respectively.

656 **Fig. 2.** (A) Confirmation of antigenicity was tested using 20 U of TAG-72 per

657 well. Five micrograms per well of purified VLP-rscFvs protein samples were

658 loaded and the wells were probed with mouse anti-DDDDK and secondary

659 antibodies were goat anti-mouse IgG for rscFvs. Black and white bars denote

660 VLP-rscFvs and VLPs samples, respectively. Data are the mean±SD (n=3)

661 (B) Presence of GPI anchor was confirmed by PI-PLC treatment by ELISA

662 method. Black and white bars represent with and without PI-PLC wells. Data

663 are the mean±SD (n=3).

664 **Fig. 3.** TEM and Immuno-TEM pictures. (A) VLP-rscFvs. Scale bar is 200 nm.

665 (B) VLP-rscFvs with 10 nm gold particles. Scale bar is 100 nm. (C) LUV-SRB.

666 Scale bar is 100 nm. (D) VLP-rscFv-SRB. Scale bar is 200 nm. (E) VLP-

667 rscFv-SRB with 10 nm gold particles. Scale bar is 200 nm. (F) VLP-rscFv-

668 SRB showing association. Scale bar is 100 nm. Black arrows denote

669 distinctive bilayer of VLPs. White arrows indicate LUVs-SRB. (G) DLS data of

670 LUV-SRB (long dash line), VLP-rscFvs (solid line) and VLP-rscFv-SRB (short

671 dash line). One hundred micrograms of VLP-rscFvs and VLP-rscFv-SRB

672 were used for assay, showing the shift in size (diameters in nm) before and

673 after association.

674 **Fig. 4.** Purified VLP-rscFv was used to qualitatively evaluate GP64 functions

675 by hemolysis assay. Rabbit erythrocytes when come in contact with GP64 a

676 pore forming protein causes an increase in porosity of the membrane leading

677 to leakage of heme into supernatant, which is measured colorimetrically.

678 **Fig. 5.** VLPs-rscFvs were observed by CLSM after treatment with Alexa594

679 conjugated anti-DDDDK (A), in bright field image (B) and the merged image

680 (C). Scale bar is 1 μm. (D) Schematic representation of VLP-rscFvs on the

681 glass slide.

682 **Fig. 6.** CLSM pictures of LS174T cells with VLP-rscFvs (A-D), with VLPs (E-

683 H), and HEK293T cells with VLP-rscFvs (I-L), respectively. (A, E, and I) DAPI

684 and bright field overlapped image. (B, F, and J) BODIPY FL-C5-ceramide

685 complexed to BSA for plasma membrane localization. (C, G, and K) Alexa594

686 conjugated anti-DDDDK for rscFvs. (D, H, and L) Merged images of all the

687 color channels. Scale bars denote 20 μm.

688 **Fig. 7.** CLSM pictures of LS174T cells with (A-C) and without (D-F) VLP-

689 rscFv-SRB treated for co-localization studies. (A and D) FITC-conjugated goat

690 anti-mouse IgG against mouse anti-DDDDK image. (B and E) SRB image and

691   (C and F) bright field plus merged images of all the color channels. Scale bars

692   denote 5 µm.

693   **Fig. 8.** The rendered color-coded depth projection of the entire z-stack of x-y

694   images (n=338) for the spheroid. (A) The schematic representation to show

695   the lens position and the Z-stacking position. (B) Color coded depth projection

696   of SRB. (C) Color coded depth projection of rscFvs. (D) Merged image of (B)

697   and (C).

698   **Supplementary Fig. 1.** Schematic representation of the hypothesis behind

699   the VLP-rscFv-SRB homing onto the cancer cells and delivery of dye to cells.

700   **Supplementary Fig. 2.** Large spheroids of 500 µm diameter with 5×7 array

701   using 3D Petri dish were prepared. Scale bars are 500µm.

702   **Supplementary Fig. 3.** CLSM pictures of LS174T cells with DAPI plus bright

703   field (A), 1 mM SRB (B) and merged images (C) of the color channels. Scale

704   bars are 20 µm.

Bright field plus DAPI

BODIPY FL-C5-ceramide

A594

Merged

FITC

SRB

Bright field plus Merged

A

B

C

With VLP-rscFv-SRB

D

E

F

With LUV-SRB

**VLP-rscFv-SRB homing
on cancer cell**

Endocytosis

Delivery of SRB

← **Plasma membrane**

VLP-rscFv-SRB →

Associated/Packaged SRB →

scFv against TAG-72 →

TAG-72 →

Cytoplasm