

# Fluorescence characterisation of multiply-loaded anti-HER2 single chain Fv-photosensitizer conjugates suitable for photodynamic therapy†‡

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We report the synthesis, spectroscopic properties and intracellular imaging of recombinant antibody single chain fragment (scFv) conjugates with photosensitizers used for photodynamic therapy of cancer (PDT). Two widely-studied photosensitizers have been selected: preclinical pyropheophorbide-*a* (PPa) and verteporfin (VP), which has been clinically approved for the treatment of acute macular degeneration (Visudyne®). Pyropheophorbide-*a* and verteporfin have been conjugated to an anti-HER2 scFv containing on average ten photosensitizer molecules per scFv with a small contribution ( $\leq 20\%$ ) from non-covalently bound molecules. Confocal fluorescence microscopy demonstrates good cellular uptake of PPa conjugate with the HER2-positive cell line, SKOV-3, while negligible cell uptake is demonstrated for the HER2-negative cell line, KB. For the VP conjugate, increased rate of cellular uptake and prolonged retention in SKOV-3 cells is observed compared to free photosensitizer. In clinical applications this could provide increased potency and desired selectivity towards malignant tissue, leaving surrounding healthy tissue unharmed and reducing skin photosensitivity. The present study highlights the usefulness of photosensitizer immunoconjugates with scFvs for targeted PDT.

## Introduction

Photodynamic therapy (PDT) is being developed alongside surgery, radiotherapy and chemotherapy as a minimally invasive procedure for cancer treatment.<sup>1</sup> PDT is based on the light activation of sensitizer molecules, which are preferentially localised in the target tissues<sup>2</sup> and thus its application is limited to tumours accessible to external irradiation, such as non-melanoma skin, head and neck, prostate and bladder cancers.<sup>3</sup> In addition to oncology, PDT has also found a great deal of success in the treatment of acute macular degeneration (AMD)<sup>4</sup> and a range of bacterial infections.<sup>5</sup>

Following activation with light in an oxygen-rich environment, photosensitizers produce reactive oxygen species (ROS, singlet excited state of molecular oxygen  $^1\text{O}_2$ , or radical species *e.g.*  $\text{OH}^\cdot$ ,  $\text{O}_2^{\cdot-}$ ), which effectively destroy the tissue *in situ* at locations where they have been produced.<sup>2,6</sup> In order to achieve the desired therapeutic effect, this procedure should leave the surrounding healthy tissue unharmed and thus production of ROS should be confined to diseased tissue. PDT treatment can provide a dual

modality of controlling selectivity: (i) by preferential retention of photosensitizer molecules in a tumour and (ii) a selective application of light. Many systemically administered photosensitizers are known to preferentially localise in the tumour compared to normal tissue. The precise mechanism of such selectivity is not clear, but might be due to poorer lymphatic drainage of the tumors, higher proliferation rates of tumour cells or leaky vasculature.<sup>1,3</sup> However this selectivity may not be sufficient as even successfully-treated patients often suffer from prolonged skin sensitivity caused by retention of photosensitizer in the skin and subsequent exposure to ambient light. On the other hand, the desired selectivity utilising precise application of light to diseased tissue might not be possible particularly during the PDT treatment of pathologies in complex anatomical sites where several organs could be simultaneously exposed to irradiation, such as found in the peritoneal cavity or prostate.<sup>3</sup>

To overcome these shortcomings different approaches are being developed, including using targeted macromolecular conjugates that employ cell type-specific localisation by ligand-receptor recognition to allow higher selectivity of photosensitizer towards its desired target.<sup>7</sup>

This approach utilises monoclonal antibodies (MAbs) as targeting moieties, directed against antigens or receptors over-expressed in cancerous cells of a particular type, *e.g.* anti-epidermal growth factor receptors (EGFR or HER2) which are found on breast and ovarian cancers.<sup>8</sup> Photosensitizers can be attached either directly onto the antibody<sup>9</sup> or *via* a polymer carrier.<sup>10</sup> The drawbacks of both of these approaches are that they involve a complicated synthesis of the conjugates, transport barriers, slower pharmacokinetics and loss of activity by either MAbs (reduced affinity) or photosensitizer (compromised photophysical properties leading to poor PDT efficiency).<sup>9-11</sup> In addition, to

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date only low photosensitizer direct loading ratios on MAbs have been reported,<sup>12</sup> rendering such approach inefficient for practical PDT applications. Alternative targeting moieties include peptides which mimic natural ligands or antibody fragments.<sup>13</sup> The main problem with this type of approach remains limited reproducibility in conjugate synthesis and low photosensitizer loading ratios.<sup>14</sup>

We have developed an effective synthetic pathway towards single chain fragment (scFv) conjugates with porphyrins. Here we report synthesis of conjugates with two photosensitizers widely used for *in vitro* and *in vivo* PDT, pyropheophorbide-*a* (PPa) and verteporfin (VP). The antibody used in this study was C6.5, an anti-HER2 scFv derived by phage display.<sup>15</sup> HER2 internalises<sup>16</sup> and is highly over-expressed on many epithelial cancers which are of relevance to PDT therapy.<sup>17</sup> We report a detailed study of the fluorescence properties of these immunoconjugates and demonstrate low non-covalent binding of photosensitizers to scFv, which compared favourably with existing literature data.<sup>14</sup> Intracellular imaging in comparison with free photosensitizers demonstrate cell type specificity of the former, which in combination with low non-covalent binding is a prerequisite for targeted PDT modalities and improved PDT efficacy in both *in vitro* and *in vivo* studies. We also investigate the rate of uptake and retention of VP conjugate and compare it to free VP, which was shown to rapidly diffuse from live cells shortly after the removal of the incubation medium.<sup>18</sup> This work forms the basis for testing of new scFv conjugates as photo-immunotherapeutics for *in vitro* and *in vivo* PDT which has shown promising results.<sup>19</sup>

## Experimental

The human tumour cell lines (SKOV-3, KB) were obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, penicillin and streptomycin antibiotics and passaged when 70–90% confluent in 75 cm<sup>2</sup> flasks grown at 37 °C in 5% CO<sub>2</sub>.

### Expression and purification C6.5 scFv

C6.5 scFv was obtained from Professor J. Marks (University of California, San Francisco) in pUC119 and expressed in XL1 blue cells.<sup>20</sup> The C6.5 scFv has 13 lysine residues and as such can potentially bind to the maximum of 13 photosensitizer molecules. Also the C6.5 scFv was engineered to remove lysine-100 in the antibody binding site. This was to reduce the possibility of forming PICs of reduced immuno-reactivity.<sup>21</sup> *E. coli* XL1 blue/pUC119-C6.5 cultures of 500 ml of 2TY media containing 100 µg ml<sup>-1</sup> ampicillin were grown at 30 °C and induced at an optical density (600 nm) of 0.7 by adding IPTG to a final concentration of 1 mM. Expression was allowed to continue overnight at 30 °C. The supernatant was recovered and dialysed/concentrated into PBS (phosphate-buffered saline, pH 7.4) before being purified by IMAC (immobilised metal affinity chromatography) on Talon<sup>®</sup> resin according to manufacturer's instructions. Purified protein was either concentrated to 1 mg ml<sup>-1</sup> protein using Amicon<sup>®</sup> spin concentrators and stored in 10% glycerol at –80 °C, or used for couplings straight after purification without concentrating.

### Synthesis of pyropheophorbide-*a* succinimidyl ester

Pyropheophorbide-*a* succinimidyl ester was synthesised for coupling to the scFv as follows. To a light protected solution of the pyropheophorbide-*a* (50 mg, 0.094 mmol) in a mixture of dry DCM–THF (9 : 1), *N*-hydroxysuccinimide (12.9 mg, 0.11 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (23.2 mg, 0.11 mmol). After stirring for 12 h (at room temperature) under argon, the precipitated dicyclohexylurea was filtered off and the solvents removed. The crude product was taken up in a small volume of chloroform and precipitated by the addition of hexane. The precipitate was collected, washed well with hexane and the resulting crude product purified by column chromatography on silica gel eluting with 20% hexane in ethyl acetate ( $R_f = 0.66$ ). The isolated product was re-crystallised from DCM–hexane to give pure succinimidyl ester in 70% yield.

### Synthesis of benzoporphyrin derivative (verteporfin) succinimidyl ester

The verteporfin succinimidyl ester was prepared as described for PPa. To a light protected solution of verteporfin (6 mg, 0.0084 mmol) in anhydrous THF (5 ml), *N*-hydroxysuccinimide (3 mg, 0.0261 mmol) was added followed by DCC (6 mg, 0.0291 mmol). The reaction mixture was stirred for 12 h (at room temperature) under argon when TLC (silica gel: ethyl acetate) confirmed consumption of all the starting material. The reaction mixture was evaporated and the residue purified by column chromatography on silica gel. The crude was loaded on to the column dissolved in the minimum of DCM and eluted with ethyl acetate ( $R_f = 0.74$ ) to give pure verteporfin succinimidyl ester in 75% yield.

### Synthesis of scFv-photosensitizer photo-immunoconjugates (PICs)

The photosensitizer succinimidyl ester was resuspended in 100% DMSO and added at a concentration of 52.8 µM to 3.3 µM (100 µg ml<sup>-1</sup>) scFv in PBS containing 6% acetonitrile and with continuous stirring at 40 °C for 60 min. The ratio of DMSO–acetonitrile–PBS was optimised to achieve the maximum solubility of both scFv and hydrophobic photosensitizer. We find that the high solubility of both reagents is critical for obtaining reproducible high conjugation ratios.<sup>19</sup> The photoimmunoconjugates (PICs) were then dialysed against PBS with two buffer changes. PICs were further analysed by SDS-PAGE transferred using a semi-dry blotting apparatus (Biorad) onto nitrocellulose and gently dried. Fluorescence was visualised by exciting the PPa on the blot on a short wavelength UV-transilluminator. Densitometry was used to roughly estimate the amount of free and covalently coupled sensitizer. As an example of a calculation to determine the PPa: scFv ratio, the absorbance of 65 µg ml PPa give 1 a.u. at 670 nm. Thus 0.2 a.u. is equal to 13 µg ml<sup>-1</sup> PPa which is equal to  $2.4 \times 10^{-5}$  M PPa (MW = 535). This was found coupled to a scFv at a concentration of 50 µg ml<sup>-1</sup>, which is equal to  $1.7 \times 10^{-6}$  M (MW = 30 000). Thus the ratio works out to be 14.1 : 1. Similar calculations and ratios were obtained for VP-conjugates using the maximum absorbance wavelength of 695 nm. The ratios corrected for non-covalent binding have been calculated after the precise percentage of non-covalently bound photosensitizer was established by fluorescence titrations, see below.

C6.5-VP and C6.5-PPa solutions were kept in phosphate buffered saline (PBS) at room temperature in the dark and used without further dilution for cell incubation or diluted ten times for fluorescence spectroscopic studies. Free sensitizers VP and PPa were dissolved in dimethylsulfoxide (DMSO) and small aliquots of the stock solution were added to PBS to obtain aqueous solutions of absorbance matching that of C6.5-VP and C6.5-PPa. The content of DMSO in water was less than 0.1%. Absorption at excitation wavelength (400–450 nm) of all solutions was not more than 0.1.

### Confocal fluorescence microscopy

Imaging was performed using a confocal laser scanning microscope (Leica TCS SP2), coupled to a CW argon-ion laser (488 nm). The fluorescence emission of photosensitizers from cells was spectrally dispersed using a prism and detected using a PM tube. The fluorescence spectra from cell cultures were obtained on the microscope with *ca.* 10 nm resolution. Dry 20 $\times$  (NA = 0.5) or 40 $\times$  objectives (NA = 0.75) were used in all measurements.

SKOV-3 and KB cells were seeded at 10<sup>5</sup> cells well<sup>-1</sup> in 1 ml of culture medium in untreated 1 cm coverslips placed on a 24 well plate and allowed to grow to confluence over at least 24 h. The culture media was replaced with the PBS solution containing photosensitizer conjugates and incubated for 30 min. Following incubation, the coverslips were washed twice with PBS, fixed with 4% formaldehyde solution for 1 h at room temperature and mounted on the microscope slides for imaging. For C6.5-VP conjugate uptake and release experiments the cells were seeded in untreated 8 well coverglass chambers (Lab-Tek<sup>TM</sup>, Nunc) and allowed to grow to confluence for 24 h. The chambers were placed on the microscope stage at 25 °C and the media was replaced by media containing either free VP or C6.5-VP and fluorescence images were recorded as a function of time. In a separate experiment where the cell cultures were incubated with VP (for 2.5 h) or C6.5-VP (for 30 min), the incubation media was replaced by C6.5-VP/VP-free media. The mean fluorescence intensity from resulting images was plotted to determine the rate of uptake and release of VP and C6.5-VP from cells.

## Results and discussion

### Coupling ratios of PICs

The high coupling ratios of photosensitizer to scFv were achieved due to the carefully balanced composition of the solvent reaction mixture to aid solubility of both scFv and hydrophobic photosensitizers, as discussed by Bhatti *et al.*<sup>19</sup> The other factor is the structure of the scFv, which needs to have the maximum number of available lysine residues to achieve high coupling ratios. Our results compare favourably to the previous work using the active ester coupling and L19 human antibody with 5 available lysines<sup>13</sup> and the isothiocyanate coupling described by Staneloudi *et al.*<sup>14</sup>

### Fluorescence spectroscopy of PICs

The absorption spectra of PPa and VP do not change significantly upon coupling with C6.5. Therefore we have used the absorbance at 670 nm (PPa) and 695 nm (VP) compared to a standard curve of free photosensitizers in PBS, to determine the number of

photosensitizer molecules attached to the scFv. We found that the coupling ratio of each photosensitizer to C6.5 was approximately 14:1. However this estimate does not take into account the possibility of non-covalent binding between the photosensitizer molecules and scFv. We have used fluorescence spectroscopy to investigate this further.

Normalised emission spectra of free photosensitizers compared to C6.5-conjugates are shown in Fig. 1(a) and 1(b). The spectral shape and emission maximum does not change significantly upon conjugation, however emission intensity is affected with C6.5-PPa being *ca.* 2 times more emissive than free PPa, see Fig. 2(b) for [BSA] = 0. Comparison of PPa absorption spectra in water to that in organic solvent indicates that in aqueous solution PPa is present in aggregated form. We attribute the enhancement in emission intensity in C6.5-PPa to improvement in the separation between PPa units which effectively reduces aggregation. On the other hand free VP shows more intense emission in aqueous solution than free PPa (fluorescence quantum yield for VP is 0.05<sup>22</sup> compared to 0.01 for PPa measured in this work) and this is consistent with better solubility of VP in water leading to reduced aggregation and hence less self quenching. Emission intensity is reduced approximately 3 times in a C6.5-VP conjugate compared to free VP, see Fig. 3 for [BSA] = 0. This observation is typical for the large conjugates containing several photosensitizers in close proximity<sup>21</sup> which promotes non-radiative deactivation

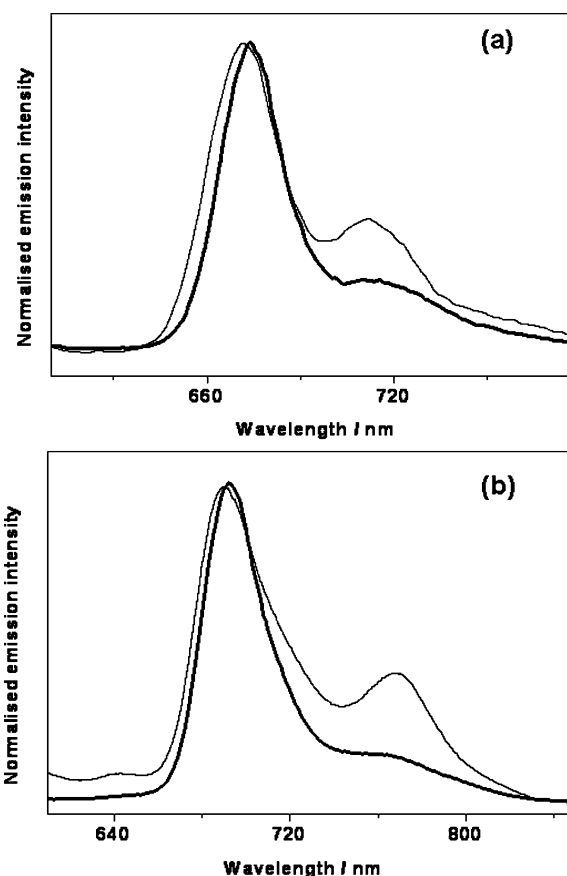


Fig. 1 Fluorescence spectra of free photosensitizers (thin lines) compared to corresponding photoimmunoconjugates (bold lines) in PBS: (a) pyropheophorbide-*a* (PPa) and C6.5-PPa, and (b) verteporfin (VP) and C6.5-VP.

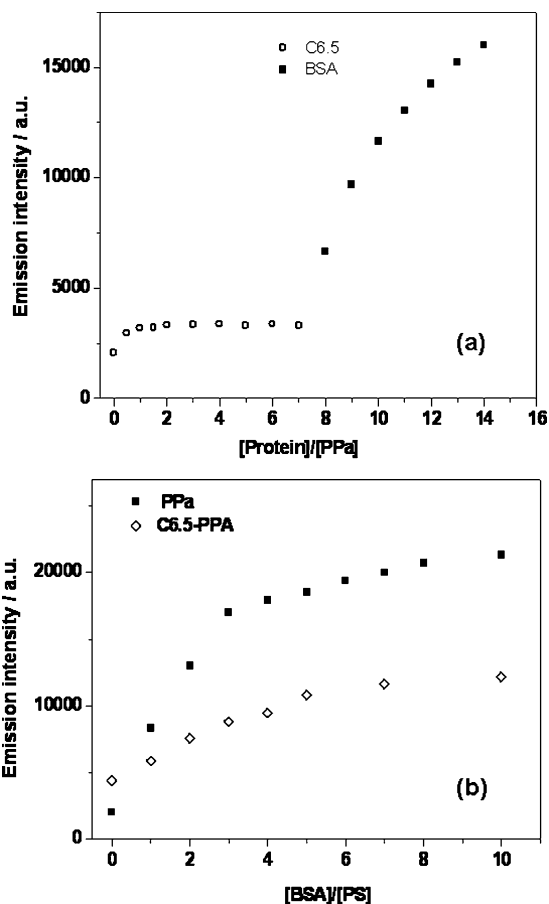


Fig. 2 Changes in the integrated fluorescence intensity observed following (a) addition of up to 7 equivalents of C6.5 (○) followed by up to 7 equivalents of BSA (■) to the solution of free PPa in PBS; (b) addition of BSA to free PPa (■) and C6.5-PPa (◇).

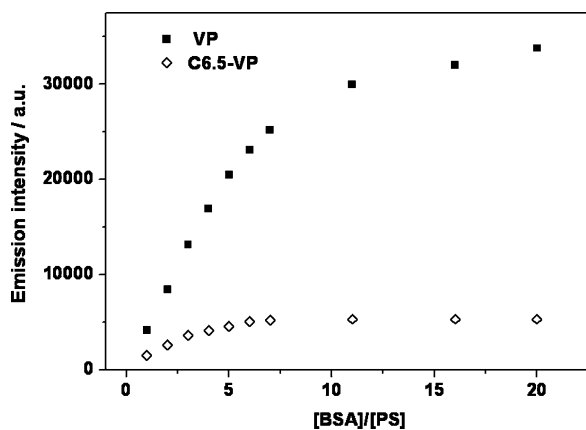


Fig. 3 Changes in the integrated fluorescence intensity observed following addition of BSA to free VP (■) and C6.5-VP (◇).

via self quenching. This phenomenon might be viewed as a 'carrier-induced' aggregation of the photosensitizer and results in compromised fluorescence efficiency.

We have monitored the fluorescence of PPa in aqueous solution upon addition of equivalents of neat scFv, Fig. 2(a). The small increase in fluorescence intensity is observed up to a 2:1

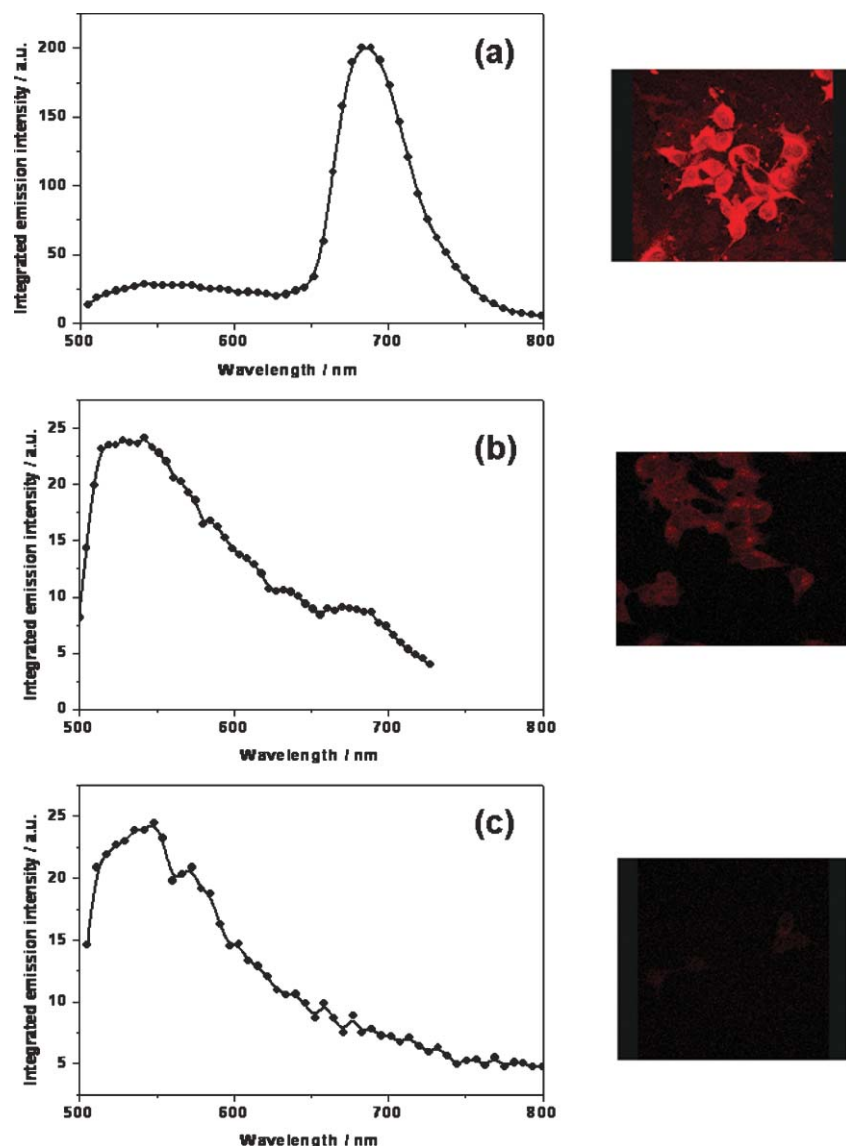
[C6.5]/[PPa] ratio. Such changes were previously reported for hydrophobic sensitizers in aqueous medium upon addition of proteins and are thought to be due to the formation of a non-covalent complex between the protein and the photosensitizer, leading to reduced aggregation.<sup>23</sup> Only a small increase in fluorescence intensity is observed in the case of adding of C6.5, consistent with a small size of scFv leading to weak hydrophobic interactions with photosensitizer and hence limited protection from self-quenching. A considerably larger effect is observed upon addition of the Bovine Serum Albumin (BSA) to free PPa in aqueous solution, indicating much stronger non-covalent interactions between PPa and BSA. BSA is widely known to contain hydrophobic pockets which interact with a range of drug molecules,<sup>24</sup> particularly in carrying anaesthetic molecules in the blood.<sup>25</sup> This conclusion is supported by the marked increase in the fluorescence intensity of the [C6.5]/[PPa] = 7 mixture upon addition of increasing amounts of BSA, Fig. 2(a). We conclude that the interaction between PPa and BSA is considerably stronger than between PPa and C6.5. Thus addition of BSA to non-covalent complex of PPa and C6.5 results in a change of carrier and this process can be monitored using emission spectroscopy. We can utilise this effect to determine the presence of non-covalently bound sensitizer in the sample of scFv PPa conjugate.

The change in emission intensity of C6.5-PPa following addition of increasing concentration of BSA is compared to the change recorded for free PPa in Fig. 2(b). The integrated intensity changes from 2400 a.u. to 20000 a.u. ( $\times 8.3$  times) for free PPa and from 4800 a.u. to 12000 a.u. for C6.5-PPa. If we assume that the emission intensity of covalently bound PPa does not change upon addition of BSA and therefore the increase in emission intensity is due entirely to non-covalently bound molecules changing the carrier, we can determine the maximum percentage of non-covalent binding as  $(12000 - 4800)/(8.3 \times 4800) = 18\%$ .

The titration curves of free VP and C6.5-VP conjugate with BSA are given in Fig. 3. Again, upon addition of BSA the emission intensity for the free VP changes from 4000 a.u. to 34000 a.u. ( $\times 8.5$  times) while for the conjugate the increase is from 1500 a.u. to 3800 a.u.. The simple calculation gives the estimated value of non-covalent binding in the sample of C6.5-VP as  $(3800 - 1500)/(8.5 \times 1500) = 18\%$ . Therefore the fluorescence data indicate that approximately 80% of the photosensitizers in C6.5 conjugates are covalently bound to scFv. This yields the coupling ratios of PPa and VP to C6.5 of around 11:1 (estimated 14:1 obtained from absorption calibration graphs, taking into account 20% non-covalent binding), which significantly exceeds the coupling ratios for the photosensitizer conjugates reported in the literature up to date.<sup>9,12</sup>

### Cellular uptake of scFv conjugates

The selectivity of the PICs to a specific cell line is essential for targeted PDT applications. To test the specificity of scFv conjugates towards HER2 receptors we investigated the cellular uptake of the scFv conjugates to HER2-positive (SKOV-3) and HER2-negative (KB) epithelial cell lines. The confocal fluorescence images of SKOV-3 and KB cells incubated with C6.5-PPa with emission monitored between 650 and 730 nm are shown in Fig. 4. While SKOV-3 cells display intense red fluorescence characteristic of PPa, KB cells show negligible fluorescence intensity in the red region



**Fig. 4** Fluorescence spectra and corresponding confocal fluorescence images (650–730 nm detection) obtained from fixed cells incubated with C6.5-PPa (a) HER2-positive SKOV-3, (b) HER2-negative KB cells and (c) KB cells incubated with the blank medium.

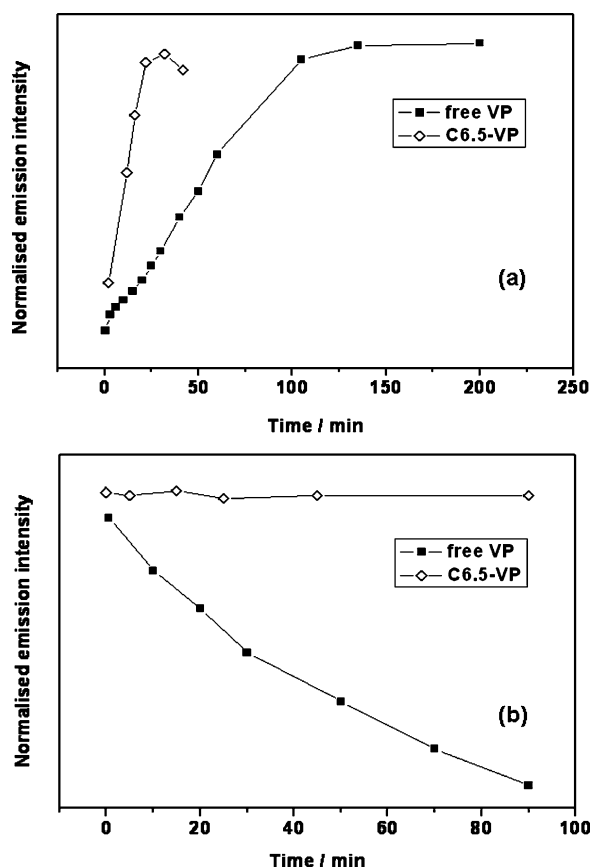
of the spectrum. The absence of red fluorescence characteristic of PPa is further confirmed by recording the fluorescence spectra of KB cells, as obtained in the case of C6.5-PPa. We have recorded only a two fold increase in the fluorescence intensity of SKOV-3 cell line compared to KB cell line (see electronic supplementary information, ESI).<sup>‡</sup> We attribute the red fluorescence signal from HER2 negative KB cell line in this case to the cellular uptake of non-covalently bound VP, which we have earlier estimated to

constitute up to 20% of the overall VP content in the conjugate. We have shown that free VP is brightly fluorescent, in particular when bound to proteins, which are abundantly present in the cellular environment. C6.5-VP conjugate shows little fluorescence and this makes the detection of C6.5-VP fluorescence from cells complicated when obscured by free VP. It is however possible to detect intracellular fluorescence from C6.5-VP unobstructed by free VP fluorescence at very short incubation times, see below.

It was previously noted that for cells incubated with VP the intracellular concentration increases fast but is significantly reduced once the incubating solution is replaced with the fresh medium.<sup>18</sup> This is in contrast to widely used clinical photosensitizer Photofrin<sup>®</sup> whose intracellular concentration remains stable after it maximises following 24 h incubation.<sup>18</sup> We have recorded the series of fluorescence images of SKOV-3 cells incubated with the VP containing medium at different time points after the incubation has started (see ESI),<sup>‡</sup> to determine the time required

to determine the time required

for the optimal cellular uptake of free VP. The mean fluorescence intensity obtained from these images is plotted in Fig. 5(a). This curve shows that the optimal incubation time for free VP is *ca.* 2 h. However, for C6.5-VP conjugate the mechanism of cellular uptake is expected to be receptor-mediated endocytosis, with significantly shorter characteristic time of cellular uptake.<sup>16</sup> The mean fluorescence intensity recorded from the images of SKOV-3 cells incubated with C6.5-VP for 30 min is shown in Fig. 5(a) and the fluorescence images recorded during the uptake processes are given in the ESI.† It is clear that the cellular uptake of C6.5-VP is much faster than that of free VP. Note the maximum fluorescence intensity for VP and C6.5-VP curves is normalised. In practice free VP intensity significantly exceeds that of the conjugate, similar to that observed in the fluorescence spectroscopy study, Fig. 3.



**Fig. 5** Time dependent change in the mean intensity obtained from the fluorescence images of SKOV-3 cells (a) incubated with free VP (■) and C6.5-VP (◇) and (b) after replacing the fluorophore containing medium with fresh DMEM medium. The maximum fluorescence intensity for VP and C6.5-VP has been normalised. Raw imaging data are given in ESI.†

Free VP leaks out of live cells when the incubation medium containing the photosensitizer is removed and replaced with normal culture medium. The series of fluorescence images of SKOV-3 cells at different time points following the replacement of VP-containing medium with the normal medium is shown in the ESI† and the mean intensity from these images is plotted in Fig. 5(b). It is clear that the amount of VP is significantly reduced after 1 h following medium replacement. The leakage of the photosensitizer from cells should not occur with C6.5-VP conjugate. Following 30 min of incubation with C6.5-VP

we washed the cells and replaced the medium containing C6.5-VP with the fresh medium and monitored the change in the mean intensity of the fluorescence images (see ESI).‡ We did not observe any decrease in the fluorescence of intracellular C6.5-VP after 90 min following the replacement of the C6.5-VP-containing medium. This result confirms our finding that VP is retained in the cells *via* HER2 specific receptor-ligand interactions and internalisation. We also note that the intracellular localisation of free VP (Fig. 6, ESI)† is different to that of the C6.5 conjugate, Fig. 5, ESI.‡ The fluorescence of free VP is concentrated mainly in the perinuclear area while the immunoconjugate is distributed more homogeneously throughout the cell.

## Conclusions

We have synthesised conjugates of PDT sensitizers pyropheophorbide-*a* (PPa) and verteporfin (VP) with a single chain antibody fragment (scFv) specific to the HER2 receptor. In both conjugates we demonstrate high coupling ratios between the sensitizers and scFv and low percentage of non-covalent binding of both sensitizers to the protein. We also demonstrate the specificity of the PPa conjugate to HER2 positive cell line, SKOV-3. In addition an increased rate of cellular uptake ( $\times 6$  times) and considerably longer retention time is observed for VP conjugate compared to free photosensitizer. We are currently exploiting the favourable properties of these new photosensitizer conjugates to achieve improved efficiency of PDT *in vitro* and *in vivo*.<sup>19</sup>

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