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Delivery of a hydrophobic phthalocyanine photosensitizer using PEGylated gold nanoparticle conjugates for the *in vivo* photodynamic therapy of amelanotic melanoma

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Dedicated to the memory of Giulio Jori; a great scientist, an excellent mentor and an even better friend.

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Abstract

Photodynamic therapy (PDT) is a treatment of cancer whereby tumours are destroyed by reactive oxygen species generated upon photoactivation of a photosensitizer drug. Hydrophobic photosensitizers are known to be ideal for PDT; however, their hydrophobicity necessitates that they are typically administered using emulsions. Here, a delivery vehicle for photodynamic therapy based on the co-self-assembly of both a Zn(II)-phthalocyanine derivative photosensitizer and a polyethylene glycol (PEG) derivative onto gold nanoparticles is reported. The PEG on the particle surface ensured that the conjugates were water soluble and enhanced their retention in the serum, improving the efficiency of PDT in vivo. The pharmacokinetic behaviour of the nanoparticle conjugates following intravenous injection into C57/BL6 mice bearing a subcutaneous transplanted B78H1 amelanotic melanoma showed a significant increase of retention of the nanoparticles in the tumour. PDT tumour destruction was achieved 3 h following injection of the nanoparticle conjugates leading to a remarkable 40% of the treated mice showing no tumour regrowth and complete survival. These results highlight that dual functionalised nanoparticles exhibit significant potential in PDT of cancer especially for difficult to treat cancers such as amelanotic melanoma.

Introduction

Photodynamic therapy (PDT) is an innovative treatment for cancer that uses visible or near-infrared light to activate a photosensitizer drug[1] to produce reactive oxygen species such as singlet oxygen,[2] which destroy the cancerous tumour.[3] The ideal characteristics of a photosensitizer for PDT are: 1) must be isomerically pure; 2) should absorb light with high efficiency thus generating high quantum yields of singlet oxygen; and 3) should preferentially interact with the cancerous cells.[4] Numerous studies have highlighted that hydrophobic phthalocyanine photosensitizers are ideal for PDT[5] since they fulfil all three criteria:[6] 1) can

be synthesised as single isomers; 2) have an intrinsic high extinction coefficient in the far-red region of the electromagnetic spectrum, which is ideal for tissue penetration; and 3) exhibit improved biodistribution within cancerous, rather than healthy, tissue. The drawback for such hydrophobic molecular photosensitizers is that they need to be dispersed within a delivery vehicle for *in vivo* therapy. Typical pharmaceutical delivery vehicles for hydrophobic molecules are emulsions such as Cremophor, although the direct introduction of an excessive concentration of the emulsion into the bloodstream can cause onset of an anaphylactic reaction.[7] Recently, the use of nanoparticle formulations to deliver the photosensitizer, either in combination with an emulsion or using water soluble nanoparticles, has become a common strategy in PDT.[8] The transport of nanoparticles to tumour tissues is facilitated by the enhanced permeability and retention (EPR) effect,[9] a phenomenon that is characteristic of solid tumours and does not occur in healthy tissue. To meet the demands of large amounts of nutrients and oxygen in tumour tissues, new blood vessels are rapidly formed that are leaky which induces the accumulation of blood plasma components, including macromolecules and nanoparticles, in the tumour.[10] Thus, the EPR effect increases the passive accumulation of nanoparticles in the tumour and is currently used for tumour-targeting in the development of new anticancer drugs.[11]

While there have been numerous studies describing the use of nanoparticles for *in vitro* PDT,[8] there are remarkably fewer studies detailing their *in vivo* PDT efficacy.[12] Nanoparticles have been used, *via* intravenous injection or intratumour injection, for the treatment of various tumours and cell lines implanted in animal models including embryonic fibroblast,[13] brain,[14, 15] head and neck,[16-18] melanoma,[19] colon,[20-22] gastric,[23] liver,[24] cervical,[25] and breast[26-28] cancer. The delivery of such nanosystems typically shows a suppression of the tumour growth when compared with tumours treated only with the photosensitizer drugs. In this field, we have investigated the efficiency, both *in vitro* and *in*

vivo, of phthalocyanine functionalised gold nanoparticles for the treatment of cancer using PDT.[29-34] Our previous *in vivo* studies used a hydrophobic zinc phthalocyanine (C11Pc) derivative that was formulated as a nanoparticle construct (C11Pc-Np) using a phase transfer reagent.[32] Since both were non-water soluble, the free C11Pc photosensitizer and the nanoparticle conjugate were injected into the animal model using a Cremophor emulsion. A direct comparison between the free C11Pc photosensitizer and the nanoparticle formulation showed that the half-life of the C11Pc in the serum was extended from 3.5 h to 6 h for the free photosensitizer and nanoparticle conjugate respectively. Additionally, it was also found that when the mice were treated with PDT 3 h after injection of the free or nanoparticle formulation of the C11Pc the mice remained tumour free for ca. 6 days. Subsequent tumour growth was shown to be appreciably faster for the mice that had received the free C11Pc photosensitizer treatment. For efficient PDT, delivery systems should be able to reduce the probability of opsonisation of nanoparticles in the bloodstream and the uptake by the reticuloendothelial system (RES). By functionalising the nanoparticles with a combination of polyethylene glycol (PEG) and photosensitizer drugs, the blood circulation time can be increased, the uptake by the RES can be reduced and consequently the accumulation of the nanoparticles in the tumours through the EPR effect can be achieved.[11] Burda and co-workers reported the use of PEG functionalised gold nanoparticles carrying a non-covalently linked phthalocyanine for *in vivo* PDT of cancer.[35-38] Since the *in vivo* efficacy of nanoparticles for PDT increases with increased blood circulation time, it is clear that the development of delivery nanosystems with long circulation times in serum is appealing to further advance this therapy of cancer.

Here we present, for the first time, the synthesis and application of gold nanoparticles (Np) stabilised by the co-self-assembly of the hydrophobic zinc phthalocyanine (C11Pc) photosensitizer and a water soluble thiol-functionalised poly(ethylene glycol) (PEG) (C11Pc-Np-PEG, Fig. 1) for the *in vivo* delivery and PDT of

amelanotic melanoma. We have chosen amelanotic melanoma since it represents 2-8% of malignant melanoma and remains a significant challenge to both diagnose and treat.[39, 40] Cutaneous melanoma are easily recognised due to the presence of characteristic pigmented areas within the tumour. However, amelanotic melanoma is a subtype of cutaneous melanoma with little or no pigmentation and therefore can be easily misdiagnosed as a benign lesion. Delays in diagnosis of amelanotic melanoma can result in patient mortality.[41] C11Pc and PEG were covalently attached to the gold surface *via* a gold-thiol bond to avoid the disassociation of the ligands from the nanoparticle surface following administration of the conjugates. The C11Pc-Np-PEG conjugates were injected into C57/BL6 mice bearing a subcutaneous transplanted B78H1 amelanotic melanoma. The pharmacokinetic investigations showed that the retention time of the conjugates in both the serum and in the tumour increased as compared with nanoparticles functionalised with C11Pc alone. The conjugates were eliminated *via* the bile-gut pathway without observable toxicity. Significantly, the irradiation of the tumours treated with the C11Pc-Np-PEG conjugates, 3 h after injection, resulted in the destruction of the tumour with 40% of the mice showing no tumour regrowth and complete survival.

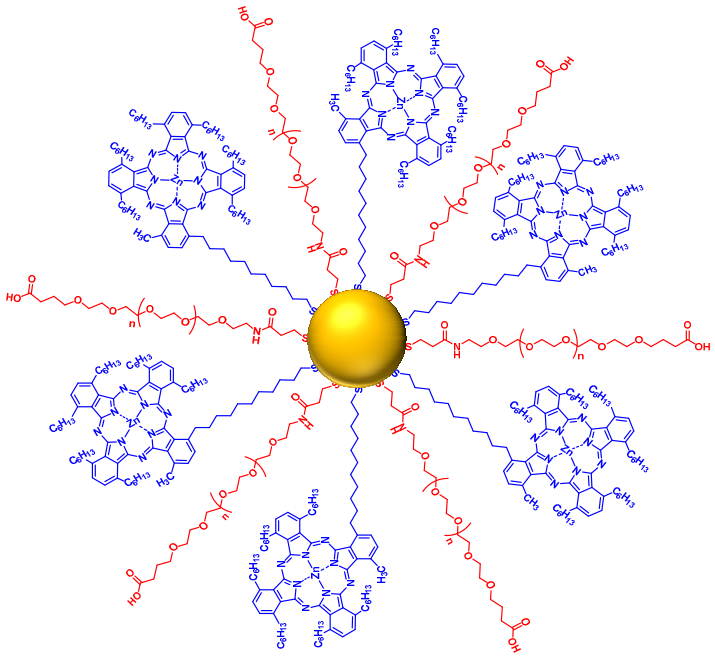

Fig. 1. Schematic representation of the gold nanoparticle with the co-self-assembled C11Pc phthalocyanine derivative (blue ligand) and the PEG (red ligand) to form the water soluble C11Pc-Np-PEG conjugates.

Experimental section

Preparation and characterisation of the phthalocyanine PEG-nanoparticle conjugates (C11Pc-Np-PEG)

The photosensitizer (C11Pc) used in this study was obtained from 1,1',4,4',8,8',11,11',15,15',18,18'-dodecakis(hexyl)-22,22'-dimethyl-25,25'-di(11,11'-dithiaundecyl)diphthalocyaninato zinc as reported previously.[33, 42] The phthalocyanine-polyethylene glycol (PEG) gold nanoparticles (C11Pc-Np-PEG) of *ca.* 4 nm in diameter were synthesised as follows: the C11Pc phthalocyanine precursor (8 mg) was dissolved in THF (4 mL). PEG (α-thio-ω-carboxy polyethylene glycol, 30 mg, Iris Biotech GmbH; 3274 Da) in THF (8 mL) was added, and the mixture was stirred vigorously for 5 min at room temperature. Gold (III) chloride trihydrate (4.8 mg in 4.8 ml of THF) was added to

the solution with further stirring for 5 min. An aqueous solution of sodium borohydride (6 mg in 4.8 mL) was added drop-wise under permanent stirring. The solution was further stirred at room temperature overnight in the dark. The solvent was removed by rotary evaporation at 60 ^{o}C, and the particles were re-suspended in MES (2-(*N*-morpholino)ethanesulfonic acid) buffer, pH 5.5 with 0.05 % Tween 20. The particles were then centrifuged in polypropylene Eppendorf tubes (1 mL) at 14000 rpm for 30 min in an Allegra X-22R centrifuge. The supernatant containing the particles was removed from the pellet with a micropipette and then characterised using UV-Visible spectrophotometry and transmission electron microscopy.

Preparation of the PEGylated gold nanoparticle (Np-PEG) – control particles

For control experiments, PEGylated gold nanoparticles (Np-PEG) were also prepared. PEG (30 mg) was dissolved in THF (12 mL), to which gold (III) chloride trihydrate (4.8 mg in 4.8 mL of THF) was added. The mixture was stirred vigorously for 5 min. Sodium borohydride (6 mg in 4.8 mL of H_2O) was added, resulting in a dark brown solution which was stirred overnight. The particles were purified using the same methods as described above for the C11Pc-Np-PEG, resulting in a light brown solution of PEGylated gold nanoparticles.

Animals and tumours

Female C57/BL6 mice (18 – 20 g body weight) bearing a subcutaneously transplanted B78H1 amelanotic melanoma were used as the animal model for these investigations. The mice were obtained from Charles River (Como, Italy) and were kept in standard cages with free access to tap water and dietary food. The procedures adopted for animal treatment and tumour transplantation were approved by the University of Padova's ethical committee for the humane treatment of experimental animals. When the tumour diameter reached a value of 0.5 cm, the mice were injected into the caudal vein with 3.0 μmol/Kg body weight of the

C11Pc-Np-PEG conjugate. In agreement with the guidelines of the Italian ethical committee for humane treatment of experimental animals, the mice were sacrificed by euthanasia when the tumour volume reached 400 mm^3, *i.e.* a size which could induce significant alterations of the animal's metabolism or the occurrence of metastases.

Pharmacokinetic studies

Healthy and tumour-bearing mice were injected into the tail vein with 3.0 μmol/Kg body weight of C11Pc-Np-PEG conjugates. At predetermined time intervals after injection, groups of three mice were sacrificed: blood samples were taken intracardiacally, centrifuged for 10 min at 3000 rpm and the sera thus collected were pooled and 50-fold diluted with THF. At the same time the tumour and selected normal tissues were rapidly excised, washed with physiological solution and a weighed amount of tissue (*ca.* 200 mg) was homogenised in 2% aqueous sodium dodecyl sulfate (SDS, 2 mL) using a Potter vessel. The homogenate was incubated for 1 h at room temperature under gentle magnetic stirring, then 0.25 mL of the suspension was diluted with THF (2.25 mL) and centrifuged at 3000 rpm for 10 min. Both the serum and the tissue extracts were assayed for the C11Pc content by reading the 640 nm-excited phthalocyanine fluorescence emission in the 660-850 nm spectral interval. The fluorescence intensity was converted into C11Pc concentration by interpolation with a calibration plot.

In a parallel set of studies, healthy mice injected with C11Pc-Np-PEG (3.0 μmol/Kg body weight) were kept in metabolic cages and the urine and faeces samples were collected and analysed for the phthalocyanine content at 24 h intervals. A weighed amount of faeces (*ca.* 200 mg) was homogenised and incubated in 2% SDS, diluted into THF and analysed for the phthalocyanine content by fluorescence measurements as described for the tissues. The C11Pc content in the urine samples was analysed using a fluorimeter after direct dilution with THF.

Photodynamic therapy studies

Irradiation of the tumour-bearing mice was performed at 3 h, 24 h and 1 week after intravenous injection of the C11Pc-Np-PEG conjugate by using the 620-700 nm wavelength range isolated by optical filtering from the emission of a halogen lamp (Teclas, Lugano, Switzerland). The light source was operated at a fluence rate of 175 mW/cm^2 for a total fluence of 157 J/cm^2 (15 min irradiation). Further details of the irradiation procedure and the approach adopted to evaluate the effectiveness of the treatment have been described previously.[32]

Results and discussion

Synthesis and characterisation of C11Pc-Np-PEG conjugates

The photosensitizer unit (C11Pc) was obtained from the precursor molecule 1,1',4,4',8,8',11,11',15,15',18,18'-dodecakis(hexyl)-22,22'-dimethyl-25,25'-di(11,11'-dithiaundecyl)diphthalocyaninato zinc as reported previously.[33, 42] To synthesise the C11Pc-Np-PEG conjugates, C11Pc and the α-thio-ω-carboxy polyethylene glycol were dissolved separately in tetrahydrofuran (THF), combined, stirred vigorously for 5 min and then gold (III) chloride in THF was added to the solution with a further 5 min period of stirring at room temperature (r.t.). An aqueous solution of sodium borohydride was added drop-wise under permanent stirring and the solution was stirred at r.t. overnight in the dark. The solvent was removed by rotary evaporation and the nanoparticles were re-suspended in MES (2-(*N*-morpholino)ethanesulfonic acid) buffer, pH 5.5 with 0.05% Tween 20. The C11Pc-Np-PEG conjugates were centrifuged, the supernatant containing the particles removed and then characterised using UV-Vis spectrometry and transmission electron microscopy (TEM). The as-synthesised water soluble C11Pc-Np-PEG conjugates were *ca.* 4.5 nm in diameter (see Fig. S1). The UV-vis extinction spectrum of the C11Pc-Np-PEG conjugates (Fig. S2) exhibits a strong absorption band at 698 nm due to the C11Pc present on the gold nanoparticle surface. Using ICP-MS, we

have established that the C11Pc-Np-PEG conjugates contain *ca.* 10 molecules of the C11Pc phthalocyanine derivative per nanoparticle.[31] For control experiments, PEGylated gold nanoparticles (Np-PEG) were also prepared as described above for the C11Pc-Np-PEG, but without addition of the C11Pc phthalocyanine derivative. Np-PEG conjugates were also characterised using TEM (Fig. S3) and UV-Vis spectrometry (Fig. S4).

Singlet oxygen production by C11Pc-Np-PEG conjugates

To confirm that the C11Pc-Np-PEG conjugates produced singlet oxygen, a buffered solution of the particles together with the singlet oxygen probe anthracene-9,10-dipropionic acid (ADPA)[43] was irradiated with a 633 nm HeNe laser for 30 min. The ADPA was photobleached over the 30 min period, confirming that singlet oxygen was produced by the irradiated C11Pc-Np-PEG conjugates. With the control nanoparticles (Np-PEG) no photobleaching of the ADPA was observed – highlighting that the C11Pc photosensitizer on the C11Pc-Np-PEG conjugates was essential for the production of singlet oxygen (Fig. S5).

Pharmacokinetic studies

The pharmacokinetic behaviour of the C11Pc-Np-PEG conjugates was assessed through the intravenous (i.v.) injection of the conjugates into C57/BL6 mice bearing a subcutaneous transplanted B78H1 amelanotic melanoma tumour. For serum and selected healthy tissues, the biodistribution of the C11Pc-Np-PEG conjugates shows that 1 week (168 h) following i.v. injection of the conjugates, the largest amounts of the C11Pc-Np-PEG, as measured by the phthalocyanine concentration, were found in the liver and spleen (Fig. 2a). This observation is perhaps not surprising since most tetrapyrrolic derivatives show a high affinity for the constituents of the reticuloendothelial system and are largely eliminated *via* the bile-gut pathway.[44] To confirm the clearance pathway followed by the nanoparticles in the mice, healthy mice were i.v. injected with the C11Pc-Np-PEG, kept in metabolic cages for 1 week, and faeces and urine samples were collected and analysed for C11Pc content at

24 h intervals. Importantly, the metabolic investigations demonstrated that no traces of the C11Pc-Np-PEG conjugates were found in the urine samples until 1 week after injection whereas large amounts of the conjugates were cleared in the faeces: thus, *ca.* 5 nmol C11Pc/gram of faeces were recovered after both 24 h and 48 h of administration of the C11Pc-Np-PEG, an additional 5 nmol/g were recovered after 96 h. Only traces of C11Pc were present in the faeces after 1 week.

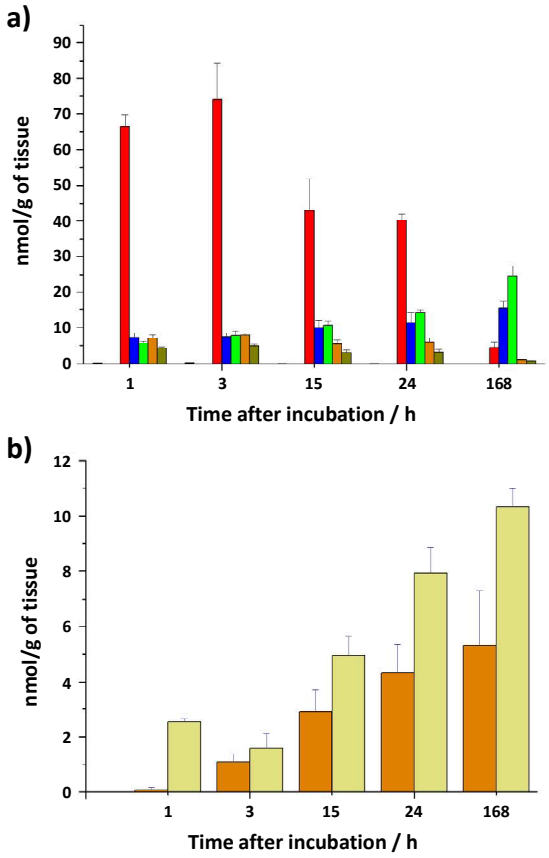

Fig. 2. Pharmacokinetic data showing the biodistribution of the C11Pc-Np-PEG conjugates following i.v. injection in C57/BL6 mice with a subcutaneously transplanted B78H1 amelanotic melanoma in: a) brain (black), serum (red), liver (blue), spleen (green), lung (orange) and kidney (dark green); and b) the amelanotic melanoma (yellow) and skin (orange).

As shown in Fig 2a, the C11Pc-Np-PEG conjugates remain in the serum at high concentrations for at least 24 h. The presence of the C11Pc-Np-PEG conjugates in the serum is in contrast to the free C11Pc photosensitizer which had a serum half-life of only 3.5

h when injected using a Cremophor emulsion.[32] The levels of the C11Pc-Np-PEG conjugates gradually decrease in serum starting with 3 h post-injection and only traces of the phthalocyanine are still present after 168 h (Fig. 2a). The apparent steady increase in the phthalocyanine recovered from the liver and spleen throughout the 1-168 h interval could give rise to some concern since accumulation of the C11Pc-Np-PEG conjugates in these organs could interfere with their metabolic processes, thus inducing toxic effects. Therefore, the pharmacokinetic investigations were extended to 8 weeks following injection using healthy mice (Table 1). The phthalocyanine recoveries from liver, spleen and serum at 24 h and 168 h are in good agreement with those measured for the tumour-bearing mice. Moreover, the total amount of phthalocyanine in the liver and spleen gradually decreases albeit at a low rate. The *ca.* 70% and 30% reduction of the phthalocyanine concentration in the liver and spleen, respectively, between 1 and 8 weeks is appreciably more pronounced than that observed for the free phthalocyanine or the C11Pc delivered by Cremophor-incorporated gold nanoparticles.[32] At the same time, no residual amount of C11Pc is found in the serum after 4 weeks, while an essentially complete disappearance is observed in lungs, brain and kidneys.

Table 1. Recovery (nmoles of photosensitizer per g of tissue) of C11Pc at selected times after i.v. injection of C11Pc-Np-PEG conjugate (3 μmol/Kg) to healthy C57 mice.

Recovery (nmol/g)				
Tissue	**24 h**	**1 w**	**4 w**	**8 w**
Skin	3.40 ± 0.82	6.93 ± 2.35	4.51 ± 0.07	3.92 ± 2.38
Serum[a]	38.90 ± 2.25	4.80 ±1.25	n.d	n.d
Liver	15.28 ± 2.25	23.24 ± 3.31	13.57 ± 0.32	8.26 ± 1.47
Spleen	16.51 ± 0.68	27.45 ± 6.18	25.18 ± 2.18	19.41 ± 2.25

[a] = nmoles per mL; n.d. = not detected

Importantly, the concentration of the C11Pc-Np-PEG conjugates recovered from the tumour steadily increases up to 1 week (168 h) post-injection and appears to exhibit a degree of selectivity in comparison to the accumulation of the conjugates in the peritumoral skin (Fig. 2b). The tumour:skin ratio of C11Pc concentration reaches values of *ca.* 2 at 24 h and 168 h. The pharmacokinetic data indicate a prolonged persistence of the conjugates in the skin that may represent a potentially negative aspect owing to the occurrence of cutaneous photosensitivity. However, skin photosensitization studies, performed by exposure of C11Pc-Np-PEG-loaded areas to light, gave no sign of the usual skin responses to photosensitized processes, *i.e.* appearance of oedema or reddening.

In vivo photodynamic therapy

The PDT of the subcutaneously transplanted amelanotic melanoma with the C11-Np-PEG conjugates was performed at 3 h and 24 h following i.v. injection, time intervals when the photosensitizer accumulates in the tumour capillaries and the neoplastic cells, respectively.[32] Therefore, irradiation at such time intervals could promote different photodamage mechanisms. To evaluate the efficacy of the PDT, changes in tumour volumes were monitored for 45 days after treatment of three groups of mice, *viz* a control group to which no C11Pc-Np-PEG were injected, and a second and third groups where mice received PDT treatment 24 h and 3 h, respectively, after injection of C11Pc-Np-PEG conjugates. The response of the amelanotic melanoma to the PDT treatment at 3 h and 24 h post-injection is highlighted in the Kaplan-Meier survival plots (Fig. 3). The survival plots show the percentage of mice whose tumour volume reached the value of 400 mm^3 as a function of post-irradiation time. The mice were sacrificed when the tumour reached 400 mm^3, following the guidelines established by the Italian ethical committee for humane treatment of experimental animals. The mice irradiated at 3 h following injection of the C11Pc-Np-PEG conjugates exhibited the largest response to PDT. All the mice in this group survived for 18 days following PDT. Importantly, 6 out of 15 mice, *i.e.*, 40%, were completely cured, as

shown by the lack of tumour regrowth up to, and including, 45 days. It is thought that the tumour response at 3 h reflects a predominantly vascular damage.[32] A similar tumour vasculature response has been observed previously using an antibody-porphyrin conjugate which was shown to selectively disrupt the tumour blood vessels.[45] Thus, our results suggest that the C11Pc-Np-PEG conjugate's photoinduced damage of the blood supply to the amelanotic melanoma lesion is of major importance for achieving a significant therapeutic result, at least for this specific tumour model.

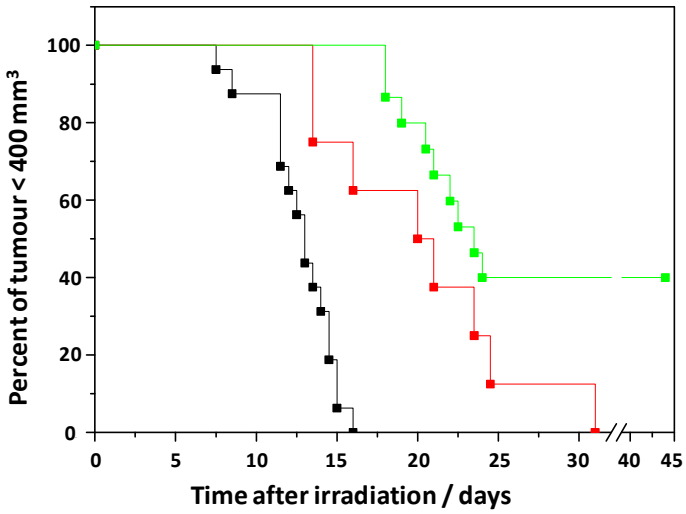

Fig. 3. A Kaplan-Meier survival plot highlighting the response of the amelanotic melanoma following PDT with the C11Pc-Np-PEG conjugates: black squares – control group (no C11Pc-Np-PEG conjugates injected), n = 15; red squares – mice treated 24 h following i.v. injection of C11Pc-Np-PEG conjugates, n = 8; and green squares – mice treated 3 h following i.v. injection of C11Pc-Np-PEG conjugates, n = 15.

The effect of PDT on the growth of the amelanotic melanoma when the irradiation was performed at 24 h after injection of the C11Pc-Np-PEG conjugates was less pronounced. While the growth of the tumour was slowed for up to two weeks in all of the phototreated mice, there was no animal survival beyond 31 days. Within the control group survival did not extend beyond 16 days. Irradiation of the tumour bearing mice was also performed at 168 h after injection of the nanoparticle conjugates. This time period corresponded to the maximum accumulation of the C11Pc-Np-PEG conjugates in the

malignant lesion (Fig. 2b). However, PDT treatment at 168 h had no therapeutic effect. It is possible that the faster rate of tumour growth is likely to reflect the appreciably larger initial volume of the tumour after 168 h (data not shown). Overall our data suggest that the efficacy of PDT using the nanoparticle conjugates is maximal when irradiation is performed at 3 h following injection when the photosensitizer concentration in the serum is significant. These results confirm that the C11Pc-Np-PEG conjugates largely perform the PDT action *via* a vascular effect.

Conclusions

In summary, we have synthesised gold nanoparticles stabilised with a monolayer of both a phthalocyanine photosensitizer C11Pc and a polyethylene glycol derivative. Despite the phthalocyanine photosensitizer being hydrophobic, the C11Pc-Np-PEG conjugates were water soluble facilitating their systemic delivery using buffered aqueous solutions. The nanoparticle conjugates were intravenously injected into mice bearing a subcutaneously implanted amelanotic melanoma. Full pharmacokinetic data highlighted the uptake of the C11Pc-Np-PEG conjugates within the melanoma and the subsequent elimination of the nanoparticles *via* the bile-gut pathway. Irradiation of the amelanotic melanoma at 3 h following i.v. injection of the C11Pc-Np-PEG conjugates induced a photodynamic destruction of the tumour. Importantly, 40% of the mice were completely cured with no tumour regrowth. These results highlight the efficacy of the *in vivo* delivery of the hydrophobic phthalocyanine using the nanoparticle conjugates for the treatment and cure of difficult-to-treat cancers such as amelanotic melanoma.

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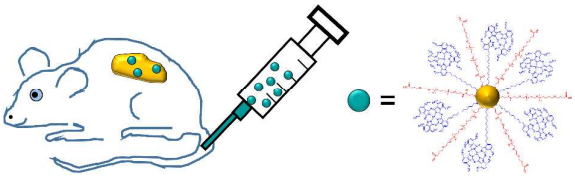
Table of Contents



Gold nanoparticles functionalised with PEG and a hydrophobic phthalocyanine photosensitizer were used for the in-vivo PDT of amelanotic melanoma. 40% of the mice treated showed no tumour regrowth and complete survival following PDT.