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Increasing the density of nanomedicines improves their ultrasound-mediated delivery to tumours

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ABSTRACT

Nanomedicines have provided fresh impetus in the fight against cancer due to their selectivity and power. However, these agents are limited when delivered intravenously due to their rapid clearance from the bloodstream and poor passage from the bloodstream into target tumours. Here we describe a novel stealthing strategy which addresses both these limitations and thereby demonstrate that both the passive and mechanically-mediated tumour accumulation of the model nanomedicine adenovirus (Ad) can be substantially enhanced. In our strategy gold nanoparticles were thoroughly modified with 2 kDa polyethyleneglycol (PEG) and then linked to Ad via a single reduction-cleavable 5 kDa PEG. The resulting Ad–gold–PEG construct was compared to non-modified Ad or conventionally stealthed Ad–poly[*N*-(2-hydroxypropyl)methacrylamide] (Ad–PHPMA). Notably, although Ad–gold–PEG was of similar size and surface charge to Ad–PHPMA the increase in density, resulting from the inclusion of the gold nanoparticles, provided a substantial enhancement of ultrasound-mediated transport. In an in vitro tumour mimicking phantom, the level and distance of Ad–gold–PEG transport was shown to be substantially greater than achieved with Ad–PHPMA. In in vivo studies 0.1% of an unmodified Ad dose was shown to accumulate in tumours, whereas over 12% of the injected dose was recovered from the tumours of mice treated with Ad–gold–PEG and ultrasound. Ultimately, a significant increase in anti-tumour efficacy resulted from this strategy. This stealthing and density-increasing technology could ultimately enhance clinical utility of intravenously delivered nanoscale medicines including viruses, liposomes and antibodies.

1. Introduction

Intravenous (IV) delivery of nanomedicines to tumours is sub-optimal due to instability in the bloodstream and poor penetration from the bloodstream and deep into tumours [1–3]. Interactions with bloodstream components have recently been more clearly defined for a range of nano-agents such as liposomes, nanoparticles and adenoviruses (Ad) [4–6]. Such studies have helped the development of chemical modification strategies which utilises polyethyleneglycol (PEG) or poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) polymers to reduce blood component binding and thereby improve circulation kinetics [7–11]. However, such chemical 'stealthing' often provides insufficient protection to allow optimal activity of these nanomedicines, or modifies them to such an extent that their activity at the target site is compromised [7,12]. Furthermore, although uptake of nano-sized agents into target tumours benefits from the enhanced permeability retention effect (EPR) [13,14], such passive accumulation still only permits 1–2% of the injected dose to accumulate. In response, device based approaches providing physical/mechanical stimuli to actively drive nanomedicines from the circulation deep into tumours have gained prominence, with application of focussed ultrasound being recognised as a particularly attractive option [15–17].

Here we describe an approach which provides enhancement of both EPR assisted passive accumulation as well as device based mechanical transport to tumours. Specifically, gold nanoparticles were sparsely modified with 5 kDa PEG and then heavily modified with 2 kDa PEG. The resulting novel 'dandelion' construct was linked via a reduction labile disulphide bond to the nanomedicine adenovirus, to give Ad–gold–PEG (see Graphical abstract). Gold nanoparticles are an ideal nanomedicine component due to their biocompatibility, low toxicity and amenability to surface modification.

Our dandelion-like structure provided a thick protective steric shield of 2 kDa PEG similar to that achieved with multivalent PHPMA-based polymers whilst still relying on one-point attachment of the

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shield to the adenovirus (via the 5 kDa PEG), as with conventional PEG coating. The disulphide bond between the adenovirus and the gold-PEG provided a mechanism for triggered de-stealthing and infection reactivation within reducing environments found within tumours [18,19]. Attachment of the dense steric shield via a minimal number of reducible 5 kDa PEG links was designed to allow effective de-shielding. Crucially, the addition of gold nanoparticles also substantially increased the density of the Ad construct, thereby enhancing its response to focussed ultrasound induced cavitation events. This strategy has the potential to broaden the clinical utility of powerful therapeutic agents by enabling their successful delivery following IV injection for cancer treatment.

2. Materials and methods

2.1. TNBS assay

A TNBS (2,4,6-Trinitrobenzenesulfonic acid) assay was used to determine depletion of amine groups on gold and Ad particles in response to addition of amine reactive PEG, PHPMA or gold–PEG [20]. In brief, standard curves of unmodified gold or Ad were treated identically to samples and TNBS added before absorbance at 335 nm was measured.

2.2. Synthesis of Ad–gold–PEG

A sample of 1×10^{14} spherical amine-presenting 5 nm gold (~300 amine groups per gold particle; Nanopartz, USA) was first conjugated to 5 kDa carboxyl-PEG-thiol (Rapp Polymere, Germany) via EDC cross-linker chemistry in 1 mL PBS (pH 4.5) at a molar ratio of 1 gold:10 5 kDa-PEG:50 EDC for 1 h at 25 °C. Amicon ultra-4 centrifugal filter (GOLD-CF; Millipore, UK) with molecular weight cut-off of 100 kDa was used to filter out excess 5 kDa-PEG. A TNBS assay was used to determine loss of amine groups from the gold surface and thereby demonstrate that on average 5 molecules of 5 kDa were attached per gold. 5 kDa-thiol-PEG–gold was later conjugated to 2 kDa methyl ether-PEG-*N*-hydroxysuccinimide (Rapp Polymere, Germany) in 1 mL PBS (pH 7.4) at a molar ratio of 1 × 5 kDa-PEG–gold:2000 2 kDa-PEG for 2 h at 25 °C. GOLD-CF was again used to filter out excess 2 k-PEG. TNBS confirmed the presence of an average of 257 molecules of 2 kDa PEG per gold nanoparticle. Gold nanoparticles fully saturated with the 2 kDa-PEG-methyl ether and 5 kDa-PEG-thiol (gold–PEG) were reduced in 1 M DTT buffer (pH 8) for 30 min to break any possible disulphide bonds formed, and purification was performed by GOLD-CF. Gold–PEG was reacted with heterobifunctional reagent SPDP in 1 mL PBS (pH 7.4) at a molar ratio of 1 gold–PEG:5000 SPDP for 1 h at 25 °C and purified by GOLD-CF. The final conjugation step was to react gold–PEG–SPDP with adenovirus-type 5 ΔE1/E3 CMV-GFP (Ad; the Native Antigen Company, UK) or Oncolytic Ad [21] in PBS (pH 7.4) at molar ratio of 1 Ad:2000 gold–PEG–SPDP for 1 h at 25 °C. Final Ad–gold–PEG particles were further purified by gel filtration on a Sepharose CL-4B column equilibrated with PBS (pH 7.4) and concentrated to 1×10^8 viral particles/μL in PBS with 10% glycerol for storage at 4 °C. The % yield for Ad was 30% to 50%.

2.3. Characterization of Ad–gold–PEG

Dynamic light scattering and zeta potential data were obtained at room temperature (RT) in PBS using Zetasizer Nano ZS instrument (Malvern, UK). For TEM analysis, Ad samples were put on formvar-coated 400 mesh copper grids (3.05 mm diameter; TAAB Laboratories Equipment Ltd, UK) for 10 min; the grids were then stained with 2% glutaraldehyde for 5 min, washed with 5 μL of ddH_2O twice, stained with 0.5% uranyl acetate for 1 min, and irradiated under the UV light (254 nm) for 10 min before being imaged under tomographic transmission electron microscope (model A-7650; Hitachi, Japan). The TEM magnification was 70,000× at the accelerating voltage of 100 kV. The processing of Ad for TEM leads to the loss of fibre protein from the capsid and so TEM gives a smaller capsid diameter compared to the hydrodynamic diameter measured by DLS. ELISA experiments were carried out as previously described [22]. In vitro experiments in which IGROV-1 and SKOV-3 were infected with Ad or Ad–gold–PEG were carried out in 96-well cell culture plates as previously described [4]. Some slight modifications included: for the IGROV-1 infection experiment, Ad and Ad–gold–PEG were exposed to reducing buffer ranging from 0 mM to 10 mM β-mercaptoethanol (BME) for 20 min; 10 mM BME was chosen as the highest level of reducing buffer because it has a reduction potential of −260 mV (pH 7) [23] which matches the reduction potentials of many reported tumour micro-environments [19,24]. GFP encoded by the Ad, and expressed in IGROV-1 or SKOV-3 cells upon successful infection, was used to measure the efficacy of infection for each sample using a FACSCalibur flow cytometer (Becton Dickinson, UK). SDS-PAGE silver staining was performed as in [7]. In vitro ultrasound experiments were performed using IGROV cells grown in 1% agar within and OptiCell pre-drilled to allow the formation of a flow channel, Ad sample and SonoVue were administered as a continuous infusion through the channel during the ultrasound exposure conditions described in Supplementary Information Fig. S4.

2.4. Synthesis of Ad–PEG and Ad–PHPMA

The synthesis of Ad–PEG [25], PHPMA, and Ad–PHPMA [26] was carried out as previously described. The characterization of PHPMA showed that it contained 6.2 M% of thiazolidine-2-thione reactive groups in side chains and had a molecular weight of 37,000 g/mol. In brief for PEGylation; 25 μL of 40 mg/mL of 20 kDa NHS-PEG in DMSO was added to 1 mL of HEPES (pH 7.4, 50 mM) with 1×10^{11} copies of Adenovirus (= 1 Ad per 300,000 PEG) for 2 h at RT. PEG of 20 kDa was used to provide a mean size similar to that of Ad–gold–PEG and Ad–PHPMA and for extended circulation in accordance with the findings of Doronin et al. [8]. For PHPMA, 20 mg/mL in HEPES (pH7.4, 50 mM) was added to 1×10^{11} copies of Ad for 2 h at RT.

2.5. In vivo bio-distribution and circulation tests of modified Ad on mouse models

CT26 or HepG2 cells were maintained with DMEM cell culture media (10% FBS). Five-week-old BALB/c female mice were obtained from the BMSU of the John Radcliffe Hospital (Oxford, UK). Each mouse was subcutaneously implanted with 5×10^5 CT26 or 5×10^6 HepG2 cancer cells into the flank. Once the tumours reached 100 to 150 mm^3, mice were dosed with 150 μL clodronate liposomes (ClodronateLiposomes.com, Netherlands). 24 h later, mice were randomly divided into eight groups of four mice, and each group was IV dosed with 1×10^{10} Ad, Ad–PEG, Ad–PHPMA, or Ad–gold–PEG. 20 μL blood samples were taken 5, 15, and 30 min after Ad injection and diluted to 200 μL in PBS. Quantitative PCR (Q-PCR) was used to detect the presence of Ad DNA in extracted DNA samples. tumour and liver samples were extracted following cull at 35 min and DNA isolated and quantified as in [4]. In all cases the quantity of adenovirus genomes in the blood, liver and tumour accounted for 95–100% of the total injected dose. In ultrasound experiments parameters and regimes were as in [17]. The ultrasound frequency and range of pressures used in the present study are comparable to those used in a number of recent studies seeking to enhance drug delivery to solid tumours other than in the brain [27]. Animal experimentation was performed according to UK Home Office guidelines and the UKCCCR Guidelines for Welfare of Animals in Experimental Neoplasia.

2.6. Statistical tests

Analyses used ANOVA followed by Newman–Keuls test for pairwise comparison of sub-groups, *, **, and *** represent p-value < 0.05, 0.01, and 0.001, respectively. Data is representative of at least two experiments.