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Localised delivery of doxorubicin to prostate cancer cells through a PSMA-targeted hyperbranched polymer theranostic

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ABSTRACT

The therapeutic potential of hyperbranched polymers targeted to prostate cancer and loaded with doxorubicin was investigated. Polyethylene glycol hyperbranched polymers were synthesised via RAFT polymerisation to feature glutamate urea targeting ligands for PSMA on the periphery. The chemotherapeutic, doxorubicin, was attached to the hyperbranched polymers through hydrazone formation, which allowed controlled release of the drug from the polymers in vitro endosomal conditions, with 90% release of the drug over 36 h. The polymers were able to target to PSMA-expressing prostate cancer cells *in vitro*, and demonstrated comparable cytotoxicity to free doxorubicin. The ability of the hyperbranched polymers to specifically facilitate transport of loaded doxorubicin into the cells was confirmed using live cell confocal imaging, which demonstrated that the drug was able to travel with the polymer into cells by receptor mediated internalisation, and subsequently be released into the nucleus following hydrazone degradation. Finally, the ability of the complex to induce a therapeutic effect on prostate cancer cells was investigated through a long term tumour regression study, which confirmed that the DOX-loaded polymers were able to significantly reduce the volume of subcutaneous prostate tumours *in vivo* in comparison to free doxorubicin and a polymer control, with no adverse toxicity to the animals. This work therefore demonstrates the potential of a hyperbranched polymer system to be utilised for prostate cancer theranostics.

1. Introduction

Prostate cancer remains a worldwide health concern, with over 600,000 cases and 200,000 men dying annually around the world [1,2]. Early stage prostate cancer typically has favourable outcomes, with a high rate of successful treatment and promising 5-year survival rates. However, late-stage prostate cancer has a significantly reduced prognosis due to a combination of many factors, including low specificity treatments in the clinic, a limited ability to diagnose in the early stages and complications related to advanced, metastatic occurrences of the disease, where the cancer has spread from the original disease site to other areas of the body [3–8]. The ability to efficiently target disease sites while simultaneously delivering treatment and diagnostic capabilities is the next crucial step towards improving on these outcomes.

Current treatments in the clinic are predominantly systemically administered chemotherapeutic drugs, external radiation and surgery [9]. While these treatments are successful in some cases, they are typically ineffective once the cancer reaches a metastatic castrate resistant stage. In the case of chemotherapeutic drugs that are delivered systemically, the toxicity to normal rapidly dividing cells such as red blood cells and immune cells is a significant problem [10]. Additionally, the poor bioavailability of most chemotherapeutics imparts a significant limitation on the dose that can be delivered to the site of interest, and can negatively impact on the

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efficacy of the treatment through reduced tumour site localisation [11,12]. However, the advantage of chemotherapy is that it is able to distribute throughout the body to treat tumours in multiple locations, whereas surgical and radiation approaches are confined to treating one area. Therefore, while both surgery and radiation can be highly effective for singular, large tumour masses, they are extremely inefficient techniques for treating metastatic cancer, and thus there remains a lack of treatment options that are both highly specific and effective for multiple tumour masses [13]. In order to overcome the current limitations, significant research effort in the area of nanotechnology is focused on improving this quality of treatment through achieving an increase in the dose localisation to tumour sites, in addition to reducing non-specific systemic toxicity.y

It is proposed that this can be achieved through promoting the selective accumulation of a carrier molecule specifically within tumour sites. It is well known that the vasculature surrounding a tumour is typically severely compromised as a result of the rapidly dividing nature of cancer cells, a phenomenon which has been exploited in nanomedicine through the Enhanced Permeation and Retention effect (EPR effect) [14]. In addition to the leaky blood vessels, poor lymphatic drainage in the area contributes to enhanced retention of circulating particles [15]. This has led to the development of nanoparticle drug delivery carriers that can achieve preferential accumulation within tumour sites, and thereby have the ability to deliver drugs in more a targeted manner. This can additionally be improved upon through the inclusion of specific targeting ligands, such as antibodies, peptides and small molecules that take advantage of specific antigenic interactions [16]. The addition of an active targeting ligand provides the drug delivery carrier with the capability for tumour cell uptake through the recognition of specific cell-surface receptors for surface binding [17]. This can translate into increased control over the final accumulation and biodistribution of the carrier following systemic injection. Both passively and actively targeted particles are likely to accumulate in the tumour site through the EPR effect, however, following localisation within the interstitial space, particles with an appropriate active targeting ligand will then recognise their receptor target, bind, and be retained within the tumour environment. Additionally, if the target receptor is internalising, this is then followed by internalisation into the cells [18]. This is an especially important consideration for drugs that act intracellularly, indicating that internalisation is mandatory for an efficient therapeutic effect, such as in gene delivery.

Utilising a biocompatible 'stealth' polymer carrier is an approach towards this that has been widely investigated [19–22]. The construction of biocompatible nanoparticles with a PEG coating for example, provides a carrier vehicle with long circulation times *in vivo* through both an increased size, and the ability to evade clearance from the bloodstream by the mononuclear phagocyte system (MPS) [23,24]. There exist many examples in the literature in which nanoparticles have demonstrated their versatility through functionalisation with various components for imaging, targeting and drug delivery, such as nanoparticles featuring active targeting ligands and encapsulating therapeutic drugs [25,26]. A further advantage of utilising a polymer based system is that the circulation times and clearance mechanisms of the nanoparticles are able to be tuned through manipulation of the properties of the carrier material, for example to allow long-term sustained released through controlled erosion, or capsule degradation at the active site through the use of biodegradable monomers [27–30].

The other important aspect that must be considered is how the drug will be incorporated into the nanoparticle. Several early examples of drug delivery nanoparticles feature the drug cargo encapsulated within a central cavity, such as within a liposome or a vesicle [31]. However, this method does not allow for precise control over the encapsulation and release of the drug, and physically encapsulated drugs can also be prone to leakage from the carrier while in circulation [32–35]. An alternative design is to tether the drug to the nanoparticle through a covalent linkage. While attachment of drugs to nanoparticle carriers through covalent chemistry requires more sophisticated synthetic requirements, the resultant system will typically be more stable to unwanted burst release of the drug, can facilitate a higher loading efficiency, and provides additional control over both the rate of the release and the site of drug release *in vivo* [36–38]. The release of the drug from the nanoparticle can be triggered through degradation of the covalent attachment, which can be achieved through a range of biological environments present in the target location. Typical *in vivo* conditions that are exploited for this purpose are the acidic environment of a tumour, the acidity of the endosome, and the enzymatic environment of the cell cytosol [39–43]. An actively-targeted nanoparticle will be localised within endosomes following endocytosis, which have pH ranges of ~4.5–6.5, providing opportunity for stimuli-responsive release through acid-labile linkages [44]. There are several covalent linkages that are commonly used for this purpose, including an acetal, ester or a hydrazone bond, which when used in conjunction with a targeted nanoparticle towards these environments will allow for specific release of the drug payload in the extracellular or vesicular sites [45–47].

Our group has previously described a theranostic based on a hyperbranched polymer platform with targeting capabilities for prostate cancer through a small molecule ligand for Prostate Specific Membrane Antigen (PSMA) [48]. PSMA has been identified as a specific cell-surface receptor that is overexpressed on many prostate cancers, as well as on lymph node and bone metastases of the disease, but with low levels detected on normal prostate tissues. Therefore, PSMA is considered a promising candidate for the specific delivery of nanoparticles to prostate cancer, with PSMA-targeted nanoparticles in clinical trials. Our previous work demonstrated preliminary evidence of stimuli-responsive drug delivery capabilities through the inclusion of a hydrazide monomer within the backbone of the hyperbranched polymer, which allowed for attachment of a model fluorescent drug through formation of hydrazone bonds. Here, we present further work on this system by exploring the targeted, stimuli-responsive controlled release of a chemotherapeutic through more extensive *in vitro* and *in vivo* characterisation, with efficacy of the nanomedicine as a therapeutic demonstrated in tumour regression studies in mice. The overall approach is described in Scheme 1.

2. Experimental

2.1. Characterisation methods

All NMR experiments were undertaken on a Bruker Avance 500 high-resolution NMR spectrometer. ^{1}H NMR spectra were collected for 128 scans. All ^{1}H diffusion spectra were recorded at 500 MHz at 95% gradient, ambient temperature, and 256 scans.

In vitro flow cytometry was performed on an Accuri C6 Flow Cytometer (BD Bioscience, San Jose, CA) using the 640 nm laser collecting through the 675/25 filter (Cy5).

In vitro microscopy images were acquired on a Carl Zeiss LSM 710 confocal laser scanning microscope.

In vivo optical imaging experiments were performed on an In Vivo MS FX Pro instrument (now supplied by Bruker Corporation). Cy5 images were collected with a 630 ± 10 nm excitation and 700 nm ± 17.5 nm emission filter set (f-stop 2.80, 2 × 2 binning, 190 mm FOV, 10 s exposure time). DsRed fluorescence images were collected with a 550 ± 10 nm excitation and 600 nm ± 17.5 nm emission filter set (f-stop 2.80, 2 × 2 binning, 120 mm FOV, 20 s exposure time). To provide anatomical context, fluorescence images were co-registered with an X-ray image (f-stop 2.80, 0.2 mm