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Thermosensitive dendrimer formulation for drug delivery at physiologically relevant temperatures†

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A simple and versatile dendrimer based platform to deliver therapeutic agents at temperatures within the physiological range, is reported. Lipoic acid conjugated at the periphery of the thermosensitive dendrimer formulations undergoes slow and sustained release at 37–42 °C, and rescues the cells from oxidative stress and a pro-inflammatory endotoxic agent.

Modern therapeutic interventions rely on controlled drug delivery to achieve optimal efficacy, using a stimulus that can be tailored under physiological conditions.[1] Considerable effort has been recently devoted to the development of nano-carriers in which drug release profiles could be modulated using a variety of stimuli including changes in pH, temperature, light *etc*.[2] Among these, thermoresponsive drug delivery systems have offered tremendous potential, and constitute a topical area of research.[3] Dendrimers are monodisperse hyperbranched macromolecules which are increasingly being evaluated for biomedical applications.[4] In the past few years, a series of efficient reactions commonly referred to as "click" chemistry, notably the Cu(I)-catalyzed alkyne-azide cycloaddition (CuAAC), the Diels–Alder (DA) reaction and thiol-ene coupling, have emerged in the field of macromolecule functionalizations.[5] In addition, it has recently been demonstrated that dendrimers can also be efficiently constructed in a layer-by-layer fashion, *via* a sequence of a combination of these *click* reactions.[6] An interesting feature of the dendrimers synthesized using the DA cycloaddition is their heat sensitivity, which leads to reversibility of the reaction. Cleavable dendrimers are well known in the literature.[7] They have attracted interest for biological applications, and the disassembly of most of the systems reported is triggered by hydrolytic, enzymatic or photolytic processes. Interestingly, the reversible nature of DA reactions has only been rarely exploited for drug delivery purposes,[8] and to our knowledge, has not been demonstrated in the field of dendrimers. Release of conjugated therapeutic agents from well-defined and monodisperse scaffolds, carried out under a physiological range of temperatures, can offer a tremendous advancement in drug delivery. We report herein, a divergent synthesis of dendrimers on a four-arm flexible core, *via* a sequence of convenient CuAAC and DA "click" reactions. The periphery of such a dendrimer was subsequently conjugated to multiple units of lipoic acid (LA) using orthogonal Diels–Alder "click" chemistry. Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a therapeutic agent introduced into clinics as an antioxidant, chelating agent and transcription factor regulator.[9] LA can facilitate re-establishing redox homeostasis in insulted cells by regulating intracellular antioxidants including vitamins C and D, glutathione and redox responsive transcription factors. Dihydrolipoic acid is involved in the reduction of cystine to cysteine thereby facilitating the uptake of cysteine making it available for glutathione synthesis in cellular adaptation to oxidative stress. The effectiveness of LA to rescue neural cells was demonstrated in the primary cortical cultures exposed to hydrogen peroxide (H_2O_2) and to 4-hydroxy-2-nonenal (HNE)-mediated oxidative stress. LA in this study was used as a model therapeutic agent to demonstrate that it can be efficiently "clicked" to dendrimers, and that it can be released from them *via* retro-Diels–Alder reaction (rDA) at 37–42 °C (physiological/pathological range). A detailed investigation of these nanoconjugates and the released LA, revealed that they are noncytotoxic, and protect microglial cells from oxidative stress.

Synthesis of the dendrimers was carried out using building blocks compatible with CuAAc and DA "click" reactions, to be performed in sequence. In this regard, two different types of bifunctional units containing (i) an azide and two furan rings (**AzFu$_2$**), and (ii) a maleimide and two acetylene moieties (**MaAc$_2$**) were prepared.[6] To synthesize the first generation dendrimer (**G1-Fu$_8$**, Scheme 1), **AzFu$_2$** was reacted *via* the CuAAC reaction with a core bearing four acetylene arms (**G0Ac$_4$**), at room temperature for 2 h (80% yield), or in a microwave reactor (65 °C) for 30 min (64% yield). The synthesis of the second generation dendrimer (**G2-Ac$_{16}$**), was accomplished by the reaction **G1-Fu$_8$** with an excess of **MaAc$_2$** (88% yield). Depending on the procedure used to perform

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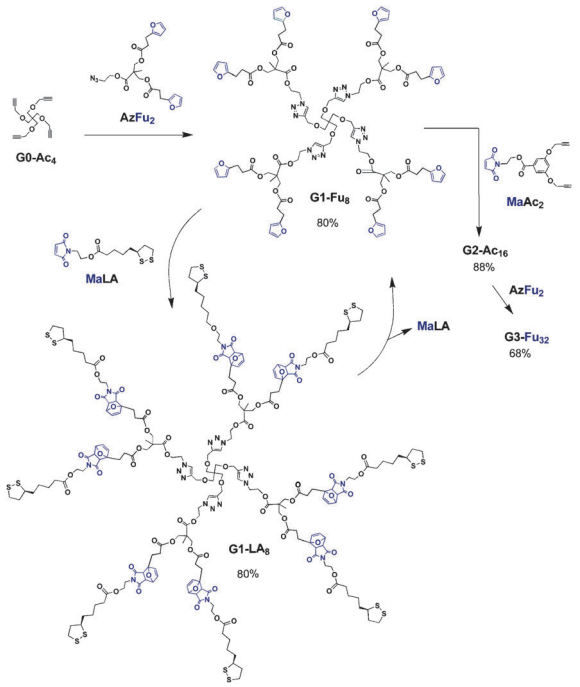
† Electronic supplementary information (ESI) available: Details of synthesis and characterization of the dendrimers as well as the materials and methods related to the biology experiments are provided. See DOI: 10.1039/c1cc15354d



Scheme 1 Synthesis of the first generation dendrimer, **G1-Fu$_8$**, its conjugation with LA to give **G1-LA$_8$**, and the preparation of the second and third generation dendrimers, **G2-Ac$_{16}$** and **G3-Fu$_{32}$**.

the reaction, different isomer ratios (*endo*/*exo*) for the DA cycloaddition were obtained. As expected, the more thermodynamically stable *exo* isomer was obtained as the major one (90%) when the mixture was heated at 55 °C in ethyl acetate for 3 days. It is interesting to note that, when the reaction was performed using this strategy, <5% of the peripheral furan moieties did not react with **MaAc$_2$**, and a subsequent one day room temperature reaction was necessary to obtain a "defect free" dendrimer. The more kinetically stable *endo* isomer was obtained in a higher proportion (50%) when the reaction was only performed at room temperature. However, 6–11 days were required for this reaction to go to completion. The reaction of **G2-Ac$_{16}$** with **AzFu$_2$**, *via* a CuAAC reaction, led to the synthesis of the third generation dendrimer, **G3-Fu$_{32}$** (68% yield). We were intrigued to note that both the second and third generation dendrimers (**G2-Ac$_{16}$** and **G3-Fu$_{32}$**) underwent rDA disassembly at ambient temperature, to a larger extent with an increase in temperature, and this behavior prompted us to explore our dendritic system for drug-delivery purposes.

In order to conjugate LA molecules to the first generation dendrimer **G1-Fu$_8$**, we designed a building block containing both a maleimide and a LA unit (**MaLA**, Scheme 1), that could be "clicked" *via* DA reaction onto the dendrimer peripheral furan moieties. The synthesis of the LA conjugated dendrimer **G1-LA$_8$** was performed using similar procedures as the ones previously discussed for the synthesis of **G2-Ac$_{16}$**, also leading to similar *endo*/*exo* DA isomer ratios. We noted that **G1-LA$_8$** also undergoes rDA disassembly, with a rate depending on the temperature, until the reaction equilibrium is reached. The time dependence of the **MaLA** release from the dendrimer was evaluated by ^{1}H NMR. Under the conditions used for the

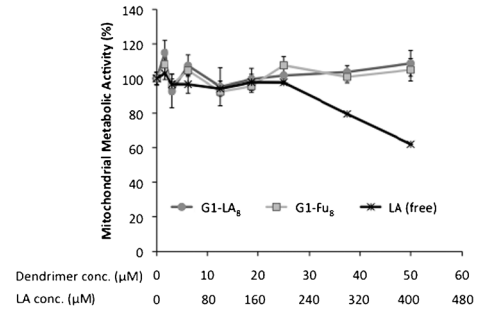


Fig. 1 Mitochondrial metabolic activity determined by MTT assay, upon exposure to **G1-LA$_8$** (0–50 μM, dendrimer conc.), **G1-Fu$_8$** (0–50 μM, dendrimer conc.) or LA (0–400 μM, LA conc.) for 24 h. Note that LA is added in equimolar concentration to that of **G1-LA$_8$**. Metabolic activity (%) in treated cells was expressed relative to controls (untreated cells that were set to 100%) (n = 9).

experiment, it was found that the equilibrium is reached after approximately three days (**MaLA** release of 10, 40 and 50% at 22, 37 and 42 °C, respectively), and that at physiological/pathological temperatures (37–42 °C), 20–30% of the **MaLA** units are released within the first 24 h.

In order to demonstrate that LA retains its biological activity when provided as **G1-LA$_8$** and to show that the dendrimer moieties are not cytotoxic, a series of experiments were carried out using living cells, *i.e.* microglia. This cell type was selected because microglia respond to even minute disturbances in the central nervous system; they efficiently patrol, survey and sense small disturbances.[10] Depending on the extent of their activation, microglia can protect the neurons, or, if excessively active, they can contribute to their cell death.

The first aim was to show that microglia remain viable and functional upon exposure to dendrimer without and with LA. Non-treated cells served as negative controls and cells treated with unconjugated LA were positive controls in mitochondrial metabolic activity assay (Fig. 1). Microglia were exposed to a large concentration range of dendrimers (0.005–50 μM) and mitochondrial metabolic activity/viability was assessed after 24 h. Dendrimers were non-toxic during 24–48 h within the tested concentration range.

G1-LA$_8$ was then tested in cells exposed to three different insults: paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), H_2O_2 and lipopolysaccharide (LPS). It was anticipated that LA released from the dendrimer would retain its biological activity and would be capable of protecting the cells from reactive oxygen species (ROS), particularly from an excess of superoxide (O_2^-) induced by paraquat (PQ) (Fig. 2A and B), H_2O_2 (Fig. 2C and D) or nitric oxide generated in microglia treated with LPS (Fig. S3, see ESI). PQ is a widely used herbicide causing damage to multiple organs (liver, kidney, heart, lungs, central nervous system).[11] It is commonly used to produce injury to dopaminergic neurons mimicking certain features of Parkinson's disease in experimental animals. In order to quantify the superoxide level within the cells upon treatment with PQ, dihydroethidium (DHE) was used. DHE oxidation by superoxide generates ethidium, whose fluorescence can be easily monitored.[12] Interestingly, the treatment of PQ-stressed microglia cells with **G1-LA$_8$**, or its precursor **G1-Fu$_8$**, resulted in a significant reduction of intracellular

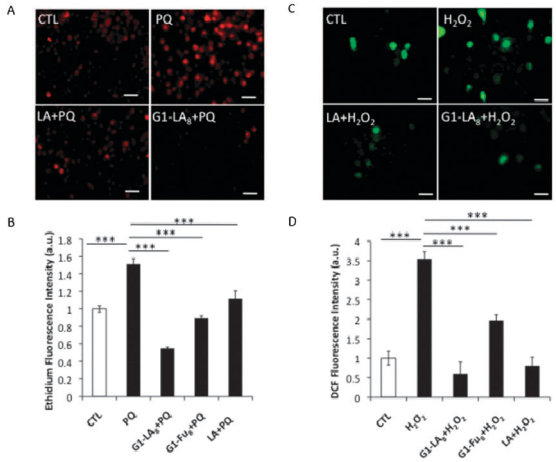


Fig. 2 Generation of superoxide and reactive oxygen species (ROS) by paraquat (PQ) and H_2O_2. Microglia were treated with **G1-LA$_8$** (12.5 μM), **G1-Fu$_8$** (12.5 μM) or LA in equimolar concentration (100 μM) for 24 h. (A) Fluorescent micrographs showing superoxide anion (O_2^-) generation following PQ exposure (500 μM, 3 h) using dihydroethidium (DHE). (B) Spectrofluorometric determination of ethidium fluorescence intensity (arb. units). Controls (CTL) = untreated cells (n = 6). (C) Fluorescent micrographs showing ROS generation following H_2O_2 exposure (200 μM, 3 h) using DCFH-DA; Scale bar, 20 *μm*. (D) Spectrofluorometric determination of DCF fluorescence intensity (arbitrary units relative to control set at 1).

superoxide (Fig. 2A and B), to an even larger extent than with initially unbound LA. The ratio between the cells labelled with ethidium (red) and the total number of cells corroborated the results obtained by spectrofluorometric superoxide determinations (Fig. 2A and B).

When microglial cells were exposed to H_2O_2, a common inducer of oxidative stress (Fig. 2C and D), a general ROS marker 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used. Intracellular DCFH-DA reacts with different ROS species yielding a highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF). The fluorescent signal was significantly reduced in cells treated with dendrimer, coupled with LA or devoid of LA. The observation that **G1-Fu$_8$** can reduce ROS (although not as effectively as a control agent LA), merits further investigations to explain the mechanisms involved in ROS suppression in microglia and other cell types.

We next examined the protective effect of dendrimer with and without LA in cells exposed to LPS, a well established endotoxin released from Gram negative bacteria, which is commonly used to mimic bacterial infection and to activate microglia. The results (Fig. S3, see ESI†) clearly show that LPS induced an increase in nitric oxide which was significantly reduced, with LA, and the dendrimer with or without bound LA (*i.e.* **G1-Fu$_8$** and **G1-LA$_8$**). We are intrigued by the activity of **G1-Fu$_8$**, and the studies to understand it in more detail are currently being pursued in our laboratories.

In summary, our studies demonstrate that the functionalization of dendrimers using Diels–Alder reaction, at ambient temperature, is a suitable strategy to prepare drug conjugates carrying multiple drug molecules which can be released under physiological (37 °C) or pathological (42 °C) conditions. The release of an active and powerful anti-inflammatory and antibacterial agent by rDA could be particularly attractive for therapeutic interventions against bacterial infections accompanied by an increased local or systemic body temperature. The fine-tuning of rDA reactions within the physiologically and pathologically relevant temperature range makes the synthesis of other drug-dendrimer conjugates widely applicable, and offers advantages over free drugs by providing better temporal and spatial drug concentration at the sites of excessive ROS production and inflammation.

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