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# Grafting of synthetic mannose receptor-ligands onto onion vectors for human dendritic cells targeting†

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**A practical preparation of onion vesicles targeted to dendritic cells involves the grafting of mannose-mimetic clusters, bearing a hydrazino group, onto the surface of onion vesicles containing an aldehyde functionalised lipid.**

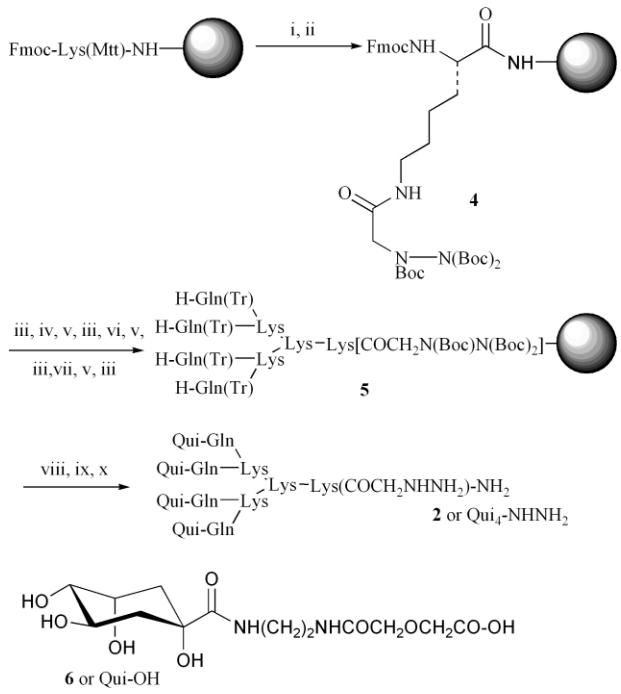
Onion vectors (or spherulites™§) are multilamellar lipidic vesicles obtained by shearing lamellar phases.[1] As they are made from pure lamellar phase with no excess of water, they contain no water core and are thus more mechanically resistent than liposomes. They are constituted of both aqueous and lipophilic compartments which can be used for the encapsulation of hydrophilic as well as lipophilic molecules in large amounts. Onions can encapsulate DNA[2] as well as proteins[3] and deliver their content to target cells *in vitro*[4] and *in vivo*.[5] To refine the controlled delivery of drugs *in vivo*, we considered the possibility of targeting neutral onion vectors to the tissues of interest by modifying their surface with recognition patterns.

To this aim we envisaged to graft molecular addresses, bearing a hydrazine function, with the aldehyde group of lipid **1**[6] (Scheme 2), already incorporated in the onion vesicles: The multilamellar nature of the onions precludes the direct insertion of a lipidic molecular address during their preparation as the address would be mostly embedded in the internal layers. As a proof of principle we elected to graft the tetraquinoylated-L-lysinyl tree **2** (Scheme 1), whose closely related constructs are ligands of the mannose receptor expressed on human dendritic cells (DCs).[7] The mannose receptor, which binds to and mediates the uptake of potentially harmful molecules or microorganisms bearing exposed mannose or fucose residues,[8] has appeared as a valuable entry for the selective delivery of bioactive material to the DCs, in particular when using mannose coated-liposome carriers.[9]

Synthesis of quinoylated tree **2** was performed on PAL-PEG-PS resin¶ preloaded with a Fmoc-L-Lys(Mtt)-OH residue using the Fmoc/*tert*-butyl chemistry[10] (Scheme 1). Introduction of the hydrazino group was secured using [*N*,*N*′-tri(*tert*-butyloxycarbonyl)hydrazino]acetic acid **3**,[11] after selective TFA removal of the 4-methyltrityl protective group[12] to give peptidyl resin **4**. Several deprotection steps and couplings with Fmoc-L-Lys(Fmoc)-OH and Fmoc-L-Gln(Tr)-OH[13] furnished the tetravalent core **5**, which was further linked to acid derivative **6**. Branched quinoylated construct **2** ($Qui_4$-$NHNH_2$) was finally obtained after acidic cleavage from the resin, sodium methoxide treatment[14] of the crude residue to hydrolyse the ester side-products formed between acid function of **6** and hydroxy groups of the quinic moieties upon coupling and RP-HPLC purification, in 15% overall yield.

Parallely, two sets of fluorescent onion vectors were prepared from PC–Sim–Rhd-DHPE–water (45.5 : 19.5 : 0.16 : 35 by weight) (control) and PC–Sim–**1**–Rhd-DHPE–water (39 : 19.5 : 6.5 : 0.16 : 35 by weight) (aldehyde) mixtures.[15] Aldehyde lipid **1** incorporated into onion vectors did not modify the structure of the vectors: indeed, the lipid mixtures appeared homogeneous when observed in polarized light microscopy and had the characteristic aspect of lipid lamellar phases. They had an usual mean size (about 200 nm in diameter, from light scattering analysis) and presented the characteristic cross when observed between crossed polarizers. The mean valency of aldehyde onion vectors was estimated to about $2.0 \times 10^4$ surface aldehyde moieties/aldehyde onion vector, as the molar ratio of **1** in the vector lipids was 6.5%, and as about 16% of total lipids belonged to the outer lipid monolayer on the basis of a lamellar phase periodicity of 75 Å.

Aldehyde (Scheme 2) and control onion vectors (4 nM = $2.5 \times 10^{12}$ vectors.ml$^{-1}$) dispersed in 10 mM acetic acid/sodium acetate buffer (pH 5), were reacted with a 2.1 fold excess of construct **2** for 16 h at 37 °C. The suspensions were then centrifuged (20000*g*, 30 min, 4 °C), to separate the supernatants containing the unreacted $Qui_4$-$NHNH_2$ and the vectors.




**Scheme 1** *Reagents and conditions*: i, TFA–$CH_2Cl_2$ 1 : 99; ii, 1.2 equiv. **3**, HBTU–HOBt–DIPEA (1.2 : 1.2 : 3.6 equiv.), DMF, 45 min; iii, piperidine–DMF 20:80; iv, 10 equiv. Fmoc-L-Lys(Fmoc)-OH, HBTU–HOBt–DIPEA (10 : 10 : 20 equiv.), DMF; v, $Ac_2O$–DIPEA–DMF 3 : 0.3 : 96.7; vi, 5 equiv. Fmoc-L-Lys(Fmoc)-OH, HBTU–HOBt–DIPEA (5 : 5 : 10 equiv.), DMF (twice); vii, 5 equiv. Fmoc-L-Gln(Tr)-OH, HBTU–HOBt–DIPEA (5 : 5 : 10 equiv.), DMF (twice); viii, 4.8 equiv. **6**, HBTU–HOBt–DIPEA (4.8 : 4.8 : 9.6 equiv.), DMF (twice); ix, TFA–$^{i}Pr_3SiH$–$H_2O$ 95 : 2.5 : 2.5, RT, 2 h; x, 12 mM NaOMe, MeOH.

† Electronic supplementary information (ESI) available: full experimental details. See http://www.rsc.org/suppdata/cc/b2/b206980f/

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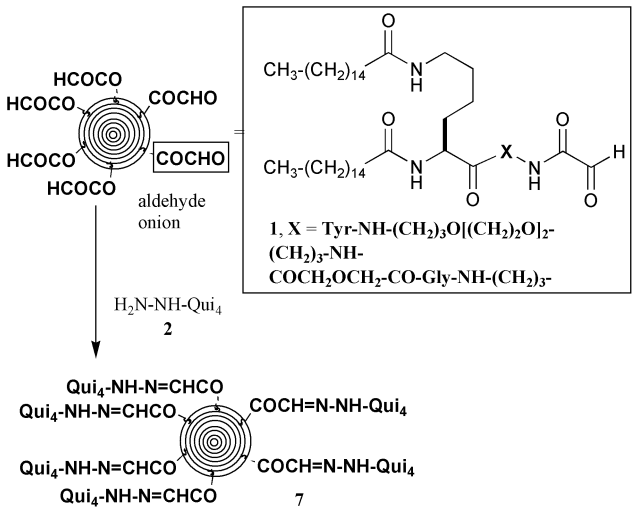
Recovered supernatants from aldehyde and control onion vectors suspensions contained 50 and 88% of the initial amount of $Qui_4$-$NHNH_2$ or derivatives thereof[16] respectively, as determined by RP-HPLC analysis: a 50% recovery of $Qui_4$-$NHNH_2$ in the supernatant of aldehyde onion vectors was consistent with a 80% yield for the ligation taking into account the reagents stoichiometry and assuming the same low, 12% rate of non-specific adsorption as the one deduced from analysis of the control onion vectors supernatant.[17]

The pelleted onion vectors were analysed by MALDI-TOF-mass spectroscopy: Mass of the the expected ligation product [*m*/*z* 3722.5 for $(M + Na)^+$] and masses corresponding to intact and degraded $Qui_4$-$NHNH_2$ were detected in the aldehyde and control onion vectors respectively, confirming the occurrence of both reaction and of minor non-specific adsorption.

To assess full availability and functionnality of the grafted tetra-quinoylated construct, uptake by DCs of the onions was studied by confocal microscopy. Co-localization with mannose receptor was clearly observed for the decorated aldehyde onion vector **7** mainly at the surface after 5 min incubation and more deeply into the DCs after 1 h incubation at 37 °C, suggesting an efficient mannose receptor uptake (Fig. 1). After 1 h incubation, the onion vector seemed to be essentially confined inside vesicles, though diffused fluorescence corresponding to rhodamine could be observed. This feature reflects probably a slight decomposition of the onion and dissemination of the components throughout the cells (Fig. 1(b)). Some internalization of control onion vector also occured. Its uptake was probably mediated by non-specific endocytosis as no co-localization with the mannose receptor could be observed (data not shown).

Alltogether these results demonstrated that chemoselective ligation could be envisaged to decorate the surface of onions: vectorization of these powerful carriers might find valuable application for the drug, gene or antigen delivery.




**Scheme 2** Structure of lipophilic aldehyde, anchor **1** and hydrazone ligation onto the surface of the onion vector.

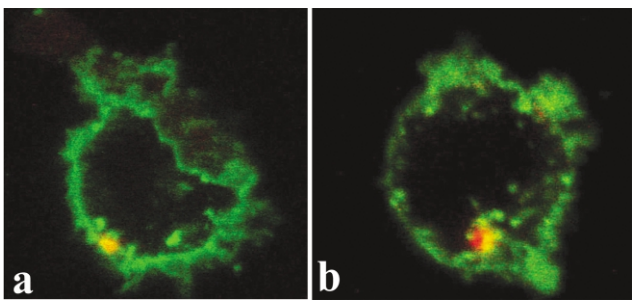



**Fig. 1** Confocal study of the cellular distribution of the decorated aldehyde onion and the mannose receptor. Dendritic cells derived from PBMC are incubated 5 min (a) or 1 h (b) at 37 °C with 200 μg $ml^{-1}$ of the decorated fluorescent aldehyde onion (rhodamine, red). The mannose receptor was detected by indirect immunochemistry using a mouse monoclonal antibody against human mannose receptor and goat anti-mouse alexa 488 antibody (green). Double exposure shows the overlapping fluorescence of the aldehyde onion **7** with the mannose receptor (yellow).

## Notes and references

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§ Spherulites™ are industrial property of Ethypharm (Pessac, France) and were used with the kind permission of the company.

¶ *Abbreviations*: PAL-PEG-PS resin, Peptide Amide Linker [5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid]poly(ethyleneglycol)–polystyrene resin; Mtt, 4-methyltrityl; HBTU, *N*-[1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxybenzotriazole; PC, phosphatidylcholine; Sim, Simulsol 2599 or 8-polyethoxy stearylester; Rhd-DHPE, rhodamine 6G-dihexadecylphosphatidylethanolamine, PBS, phosphate buffer saline.


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