

Multicellular aggregation of maltol-modified cells triggered by Fe³⁺ ions†

Alexander Ciupa, Paul A. De Bank and Lorenzo Caggiano*

Cite this: *Chem. Commun.*, 2013, **49**, 10148

Received 17th May 2013,
Accepted 12th September 2013

DOI: 10.1039/c3cc43727b

www.rsc.org/chemcomm

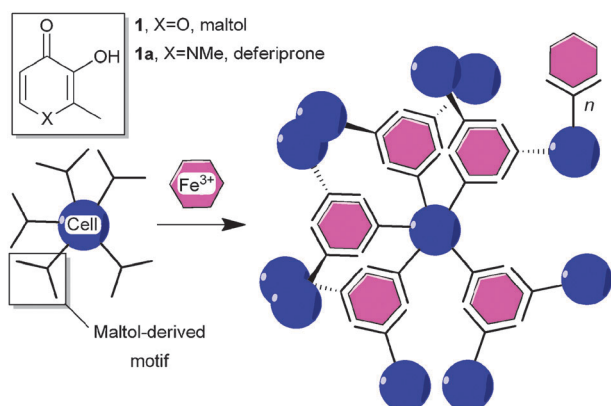
The synthesis of a maltol-derived hydrazide is described which, once attached to a cell surface, induces rapid multicellular aggregation selectively in the presence of Fe³⁺ ions. Heterocellular aggregates are also reported.

Tissues and organs are made of multiple cell types arranged in three-dimensional (3D) structures. The development of model 3D multicellular structures *in vitro* is of great interest as it has potential applications in tissue engineering¹ and as tumour models for drug testing.² A number of techniques have been used to generate 3D cell aggregates^{3–8} and we now report that ionic cross-linking, which has been extensively used to form hydrogels (*e.g.* alginate)⁹ and induce self-assembly of collagen peptides,^{10,11} can selectively generate multicellular aggregates in the presence of specific ions using cells decorated with a maltol derivative.

Maltol (3-hydroxy-2-methyl-4-pyrone, **1**, Scheme 1) is a commonly used flavour enhancer found in many foodstuffs such as beer and

bread, affording a sweet caramel-like odour.¹² It is a bidentate ligand and derivatives of maltol have found medicinal and clinical applications,¹³ such as deferiprone **1a** used therapeutically as an iron chelator.^{14–19} Macromolecular iron chelators have also been reported²⁰ and some maltol derivatives exhibit antimalarial activity²¹ and antiproliferative activity against tumour cells.^{22,23} We wished to attach maltol derivatives to cells and investigate inducing cellular aggregation through metal chelation as outlined in Scheme 1. Owing to maltol's affinity for Fe³⁺ ions, aggregation should selectively occur in their presence and not with other ions found in the cell culture medium.

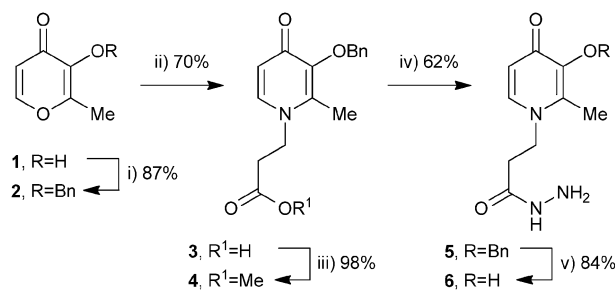
Cells have been previously modified by chemoselective ligation with hydrazides.^{4–6,24} Therefore, we explored the synthesis and use of maltol hydrazide **6** to trigger multicellular aggregation in the presence of Fe³⁺ ions. Following previous reports,^{14,15} maltol **1** was initially protected as the benzyl ether **2** (87%), which upon treatment with β-alanine gave the corresponding acid **3** in 70% yield (Scheme 2). Transformation to the *N*-hydroxysuccinimide ester^{14,15} and reaction with NH₂NH₂ was examined, although we experienced difficulties in purifying the product. Instead, conversion to the methyl ester **4** (98% yield) and simple nucleophilic substitution with NH₂NH₂ gave the hydrazide product **5** which could be readily purified by silica gel column chromatography in 62% yield. Deprotection of the benzyl ether was achieved using H₂ and Pd/C and gave the desired maltol-derived hydrazide **6** in 84% yield. Only one



Scheme 1 Schematic representation of chelate-mediated cell aggregation.

Medicinal Chemistry, Department of Pharmacy and Pharmacology,
University of Bath, Claverton Down, Bath, BA2 7AY, UK.
E-mail: l.caggiano@bath.ac.uk; Tel: +44 (0)1225 385709

† Electronic supplementary information (ESI) available: Experimental procedures, characterisation data, ¹H and ¹³C NMR spectra and additional aggregation images are available. See DOI: 10.1039/c3cc43727b



Scheme 2 Synthesis of maltol-derived hydrazide **6**. (i) BnCl, NaOH, MeOH/H₂O. (ii) β-Alanine, NaOH, EtOH/H₂O. (iii) AcCl, MeOH. (iv) NH₂NH₂, MeOH. (v) H₂, Pd/C, MeOH.



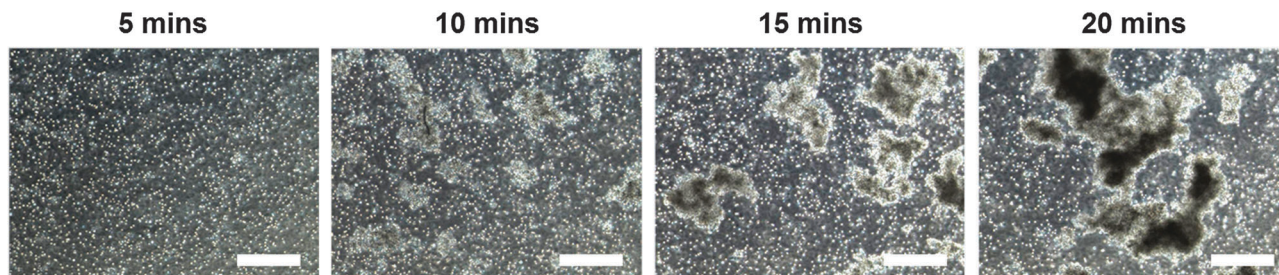


Fig. 1 Representative images of maltol-modified HT29 cells in the presence of 50 μM FeCl_3 . Scale bars = 400 μm and the results are typical of three independent experiments (provided in the ESI† together with images of unmodified cells in presence and absence of FeCl_3).

purification by column chromatography was required overall and the five-step synthesis can be readily performed on gram-scale.

With the maltol-derived hydrazide **6** in hand, we were able to investigate its attachment to cells. Human colon carcinoma (HT29) and highly metastatic human breast carcinoma (MDA-MB-231) cell lines were chosen as model cells as they are well characterised and have predictable growth properties. Since maltol derivatives exhibit antiproliferative activities with tumour cells,^{22,23} the hydrazide **6** was initially examined for activity in these cell lines using the MTS assay.²⁵ The results showed weak antiproliferative activity after 72 h in the cell lines examined, ($\text{IC}_{50} > 200 \mu\text{M}$ in HT29 and MDA-MB-231, ESI†) suggesting that further cell-based studies should be conducted at a concentration of 200 μM or below.

The HT29 cell surface was modified following previous reports using mild oxidation conditions (1 mM NaIO_4 , 10 min, 4 $^\circ\text{C}$) to generate non-native aldehydes from the sialic acid residues on the cell surface.^{4–6,24,26} The cells were then exposed to maltol hydrazide **6** (100 μM , 60 min, 20 $^\circ\text{C}$) to generate the corresponding hydrazone and thereby attaching the maltol derivative to the cell surface (see ESI† for experimental procedure). The unmodified and maltol-modified HT29 cells were resuspended in serum free medium in agarose-coated six-well plates and then treated with FeCl_3 in phosphate buffered saline (PBS, 50 μM , 20 $^\circ\text{C}$) with gentle agitation by rocking (Fig. 1 and ESI†). After 10 min, multicellular aggregation of the maltol-modified cells was observed only in the presence of Fe^{3+} , with no significant aggregates seen in the absence of Fe^{3+} or with the unmodified cells.

After 20 min of agitation, large multicellular aggregates had formed with the maltol-modified cells in the presence of Fe^{3+} which were visible to the naked eye. These results were obtained in serum-free culture medium and similar findings were also observed in PBS (not shown). However, performing the experiments in complete culture medium, containing 10% foetal bovine serum, resulted in no cellular aggregation. Presumably iron-chelating proteins such as transferrin present in the serum sequester the Fe^{3+} ions, preventing metal-mediated aggregation. Therefore, all further experiments were performed in serum-free culture medium.

Various Fe^{3+} concentrations and incubation times were examined with both unmodified and maltol-modified HT29 cells in order to optimise the aggregation process (Fig. 2). The apparent aggregate area was calculated from nine randomly selected images from three independent experiments, using previous methods.⁵ In the presence of 0, 5 and 20 μM FeCl_3 only small aggregates were observed after 20 min, although increasing the concentration to 50 μM resulted in larger aggregate sizes in only the maltol-modified HT29 cells (Fig. 2i). Increasing Fe^{3+} concentration to 200 μM resulted

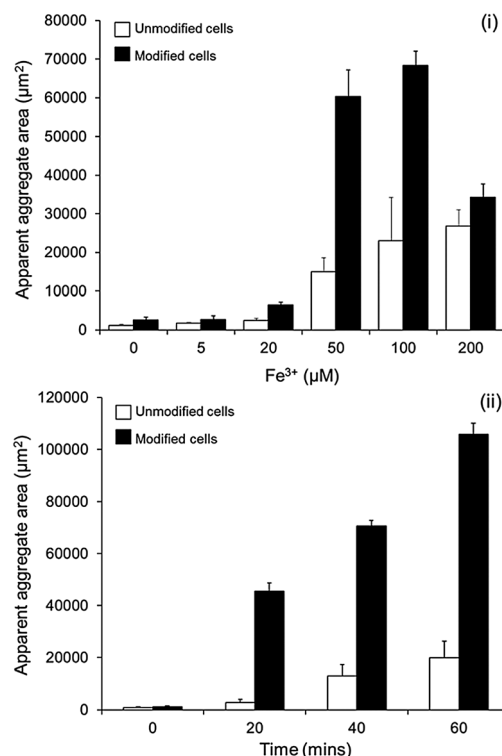


Fig. 2 The effects of (i) Fe^{3+} concentration after 20 min agitation, and (ii) agitation time with 50 μM Fe^{3+} on mean apparent aggregate area for unmodified and maltol-modified HT29 cells. Data shown as mean \pm standard error of three independent experiments.

in smaller aggregate sizes, possibly due to Fe^{3+} saturation of the maltol-derivatives. Due to toxicity associated with excess Fe^{3+} ,^{27,28} 50 μM was chosen as the optimal concentration. Time was also investigated and showed that longer times gave larger aggregates, as one would expect (Fig. 2ii). However, prolonged exposure (up to 180 min) also led to some aggregation of the unmodified cells, but only in the presence of Fe^{3+} . These aggregates were much smaller and slower to form than observed with the maltol-modified cells (see ESI†) and may be due to weak Fe-chelation by the native sugars present on the cell surface.

Although selective for Fe^{3+} , maltol derivatives can also chelate other metal ions such as Ru^{3+} ,²⁹ Cu^{2+} and Zn^{2+} .¹³ To investigate the selectivity of metal-mediated aggregation of maltol-modified cells, the metal chlorides (MCl_x) of these ions were examined under the optimised aggregation conditions (ESI†). The results demonstrate that only in the presence of 50 μM of Fe^{3+} were multicellular



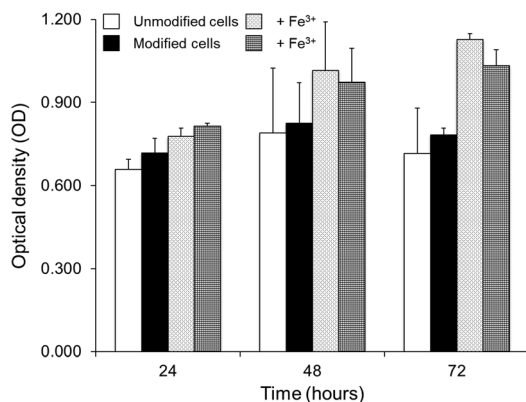


Fig. 3 Optical density of MTS assays with unmodified and maltol-modified HT29 cells in the absence (white/black) and presence (shaded) of 50 μM Fe^{3+} after 24, 48 and 72 h incubation. Data shown as mean \pm standard error of three independent experiments.

aggregates observed after 20 min, with no aggregation apparent in the presence of Ru^{3+} , Cu^{2+} or Zn^{2+} under the same conditions. The addition of potent Fe-chelators, such as EDTA or maltol **1** itself, to established aggregates did not reverse the process and aggregates appeared to be unaffected.

To examine the effects of maltol modification and exposure to Fe^{3+} on cell proliferation, modified MTS assays were performed and readings taken at 24, 48 and 72 h (Fig. 3). After 24 h, the optical density (OD) reading for the unmodified cells and maltol-modified cells were all comparable, both in the absence and presence of 50 μM Fe^{3+} . This data suggests that cells modified with the maltol-derived hydrazide are viable and, in addition, that the presence of 50 μM Fe^{3+} in both cell types did not have a detrimental effect. Similar results were also observed after 48 and 72 h.

The effects of cell aggregation using two different cell types was also examined, using human colon carcinoma (HT29) and human breast carcinoma (MDA-MB-231) cell lines functionalised with the maltol-derived hydrazide **6**. To visually differentiate the cell types, HT29 and MDA-MB-231 cells were fluorescently labelled green and red respectively using CellTracker™. The addition of 50 μM Fe^{3+} to the labelled maltol-modified cells led to the formation of heterocellular aggregates shown in Fig. 4. The HT29 (green) and MDA-MB-231 (red) cells appear randomly arranged within the aggregate giving areas of yellow, resulting from the overlap of red and green cells.

This result is of particular note as it demonstrates that modifying the cell surface with the maltol-derivative **6** and aggregation in the presence of Fe^{3+} can be applied to different cell types. Further studies will investigate possible self-organisation of heterocellular aggregates as reported by Kojima.⁸

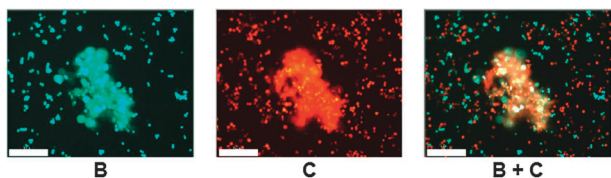


Fig. 4 Representative fluorescence images of maltol-modified HT29 cells (green, B) and MDA-MB-231 cells (red, C) in the presence of 50 μM Fe^{3+} after 20 min incubation. Scale bars = 200 μm and the results are typical of three independent experiments.

In summary, these results describe the efficient synthesis of the novel maltol-derived hydrazide **6** and its attachment to two different cancer cell lines. Preliminary experiments show that multicellular aggregation can be selectively achieved with Fe^{3+} ions, even in the presence of other ions, generating 3D aggregates on demand which could find various applications. Although cancer cells were used in this study, this approach could be applied to any cell type possessing sialic acid residues on the surface. It could be used with cells which do not naturally aggregate or to template layers of different cells to create predictable multicellular structures for use in tissue engineering.

We are grateful to the University of Bath for providing a studentship for AC and also wish to acknowledge RCUK and the University of Bath for the fellowship to LC.

Notes and references

- 1 R. Langer and J. P. Vacanti, *Science*, 1993, **260**, 920–926.
- 2 D. Yip and C. H. Cho, *Biochem. Biophys. Res. Commun.*, 2013, **433**, 327–332.
- 3 W. G. Dai, J. Belt and W. M. Saltzman, *Bio/Technology*, 1994, **12**, 797–801.
- 4 P. A. De Bank, B. Kellam, D. A. Kendall and K. M. Shakesheff, *Biotechnol. Bioeng.*, 2003, **81**, 800–808.
- 5 P. A. De Bank, Q. Hou, R. M. Warner, I. V. Wood, B. E. Ali, S. MacNeil, D. A. Kendall, B. Kellam, K. M. Shakesheff and L. D. K. Buttery, *Biotechnol. Bioeng.*, 2007, **97**, 1617–1625.
- 6 D. Zhao, S.-M. Ong, Z. Yue, Z. Jiang, Y.-C. Toh, M. Khan, J. Shi, C.-H. Tan, J. P. Chen and H. Yu, *Biomaterials*, 2008, **29**, 3693–3702.
- 7 Z. J. Gartner and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 4606–4610.
- 8 N. Kojima, S. Takeuchi and Y. Sakai, *Biomaterials*, 2011, **32**, 6059–6067.
- 9 K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869–1879.
- 10 M. M. Pires and J. Chmielewski, *J. Am. Chem. Soc.*, 2009, **131**, 2706–2712.
- 11 M. M. Pires, D. E. Przybyla and J. Chmielewski, *Angew. Chem., Int. Ed.*, 2009, **48**, 7813–7817.
- 12 D. T. LeBlanc and H. A. Akers, *Food Technol.*, 1989, **43**, 78–84.
- 13 K. H. Thompson, C. A. Barta and C. Orvig, *Chem. Soc. Rev.*, 2006, **35**, 545–556.
- 14 P. S. Dobbin, R. C. Hider, A. D. Hall, P. D. Taylor, P. Sarpong, J. B. Porter, G. Xiao and D. van der Helm, *J. Med. Chem.*, 1993, **36**, 2448–2458.
- 15 B. L. Rai, L. S. Dekhordi, H. Khodr, Y. Jin, Z. Liu and R. C. Hider, *J. Med. Chem.*, 1998, **41**, 3347–3359.
- 16 R. C. Hider, Z. D. Liu and S. Piyamongkol, *Transfus. Sci.*, 2000, **23**, 201–209.
- 17 Z. D. Liu and R. C. Hider, *Coord. Chem. Rev.*, 2002, **232**, 151–171.
- 18 Z. D. Liu and R. C. Hider, *Med. Res. Rev.*, 2002, **22**, 26–64.
- 19 T. Zhou, Y. Ma, X. Kong and R. C. Hider, *Dalton Trans.*, 2012, **41**, 6371–6389.
- 20 T. Zhou, G. Winkermann, Z. Y. Dai and R. C. Hider, *J. Pharm. Pharmacol.*, 2011, **63**, 893–903.
- 21 L. S. Dehkordi, Z. D. Liu and R. C. Hider, *Eur. J. Med. Chem.*, 2008, **43**, 1035–1047.
- 22 S. Amatori, I. Bagaloni, E. Macedi, M. Formica, L. Giorgi, V. Fusi and M. Fanelli, *Br. J. Cancer*, 2010, **103**, 239–248.
- 23 S. Amatori, G. Ambrosi, M. Fanelli, M. Formica, V. Fusi, L. Giorgi, E. Macedi, M. Micheloni, P. Paoli, R. Pontellini and P. Rossi, *J. Org. Chem.*, 2012, **77**, 2207–2218.
- 24 G. A. Lemieux and C. R. Bertozzi, *Trends Biotechnol.*, 1998, **16**, 506–513.
- 25 A. H. Cory, T. C. Owen, J. A. Barltrop and J. G. Cory, *Cancer Commun.*, 1991, **3**, 207–212, MTS is 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
- 26 K. E. Norgard, H. Han, L. Powell, M. Kriegler, A. Varki and N. M. Varki, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 1068–1072.
- 27 G. Papanikolaou and K. Pantopoulos, *Toxicol. Appl. Pharmacol.*, 2005, **202**, 199–211.
- 28 S. V. Torti and F. M. Torti, *Nat. Rev. Cancer*, 2013, **13**, 342–355.
- 29 D. C. Kennedy, B. O. Patrick and B. R. James, *Can. J. Chem.*, 2011, **89**, 948–958.

