Dunking doughnuts into cells—selective cellular translocation and *in vivo* analysis of polymeric micro-doughnuts†

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Micron-sized polymeric "doughnuts" prepared *via* dispersion polymerization were found to be highly selective in their cellular translocation abilities.

Small mono-disperse particles are valuable materials in many areas of research such as DNA sequencing,[1,2] cellular analysis, biochemical reaction multiplexing[3] as well as carriers for *in vitro* cellular sensors.[4] They are typically prepared by dispersion polymerization, a widely used approach usually carried out *via* a simple one-pot procedure using a mixture of surfactant, initiator and the required monomers.[3,5–7] It is an attractive method of polymerization due to the procedural simplicity, the ability to form mono-dispersed particles and the typically excellent yields and conversions obtained.[8,9] However, although the method is widely applied the exact mechanism is complicated and not fully understood[10] but it is believed that, following initiation, so-called "nuclei" are generated which grow by homo- and hetero-coagulation to form primary particles.[11] Swelling of these primary particles with monomer and short-chain oligomers then permits the growth of polymer spheres which eventually reach a size that allows them to "precipitate" from the reaction media.[12] Many factors are capable of altering the size and quality of the particles generated, with solvent often playing a crucial role due to its control over both monomer and polymer solubility.[11]

During investigations, mono-dispersed beads were prepared‡ for cellular uptake analysis[3] using ethanol as the major solvent with the addition of *n*-butanol, 1,2-dimethoxyethane, isobutanol, cyclohexanone and formamide all giving uniform microspheres (Fig. 1). However, an unexpected effect was observed following the addition of 5% dioxane in ethanol to the polymerization mixture with the generation, in 37% yield, of unusual particles with a narrowly dispersed micron-sized "doughnut"-like morphology (3.2 ± 0.48 μm, see ESI for details†). SEM analysis showed their doughnut-like structure (Fig. 2), which contained a 1 μm central hole. These particles maintained their morphology after re-solvation in all solvents evaluated, as well as showing the expected incorporation of aminomethylstyrene (amine loading: 9.6 μmol g^{-1}). Doughnut formation was also observed with co-solvents of diethyl-

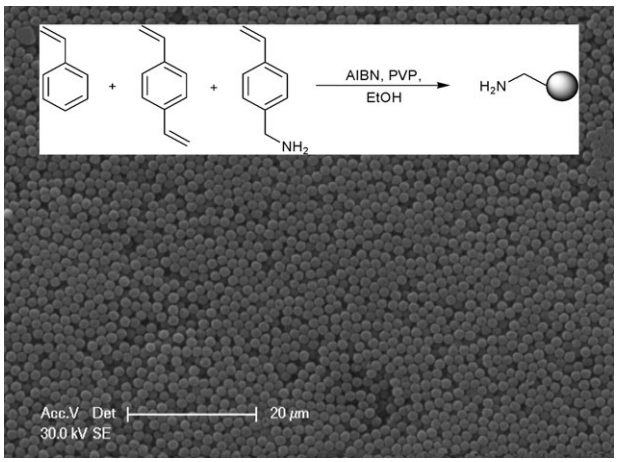

Fig. 1 SEM image of 2 μm microspheres (inset: synthetic scheme for dispersion polymerization). Scale bar is 20 μm.

ketone, isopropyl alcohol, tetrahydrofuran or toluene in ethanol, but in these cases irregular rough shaped doughnuts or only small proportions were produced (Fig. 3).

Doughnut-like structures have previously been generated using a number of techniques, such as electrohydrodynamic atomization or so-called "breath figure".[13–16] However in these cases the doughnut-like particles were typically generated within a sea of other particle types and were non-homogeneous in size and shape. Using dispersion polymerization the doughnut products were mono-dispersed and the shape was highly uniform. However, the mechanism by which micro-doughnuts are formed is not fully understood. It is clear that the introduction of dioxane to the polymerization solvent is vital; however, divinylbenzene is also thought to play a crucial role. One hypothesis is that microspheres are formed, which undergo mass transfer from the centre of the particle to the periphery, resulting in a doughnut-like morphology, analogous to the "coffee ring" effect in a three-dimensional sense. Attempts to alter the dimensions of the doughnuts *via* variation of temperature, stirring rate and the

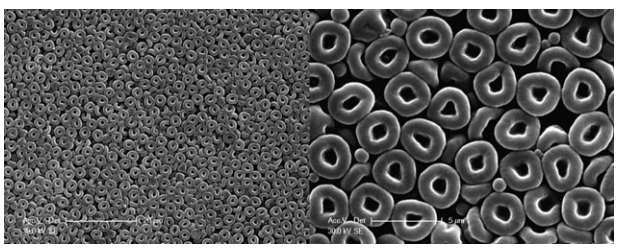

Fig. 2 SEM images of micro-doughnuts (3.2 ± 0.5 μm, scale bars are 20 μm – left and 5 μm – right).

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† Electronic supplementary information (ESI) available: Formation and fluorophore labelling of micro-doughnuts, including a confocal movie. Preparation of cell cultures and cell staining protocols. See DOI: 10.1039/b805323e

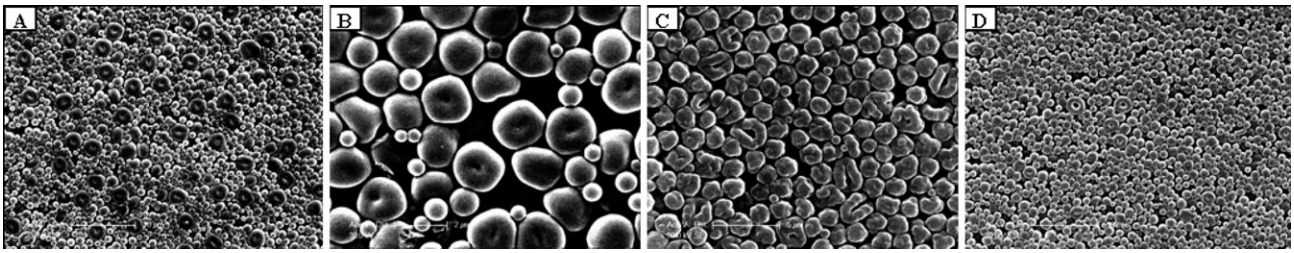

Fig. 3 SEM images of doughnuts prepared in different solvents: (A) 2% diethylketone in ethanol (scale bar is 10 μm); (B) 2% isopropyl alcohol in ethanol (scale bar is 2 μm); (C) 4% toluene in ethanol (scale bar is 5 μm); (D) 5% tetrahydrofuran in ethanol (scale bar is 10 μm).

molecular weight of the stabilizer (PVP) failed to alter the doughnut size.

The chemical accessibility of the incorporated aminomethyl groups on the doughnuts was demonstrated by labelling with carboxyfluorescein using standard coupling chemistry *via* an aminohexanoic spacer (see ESI for details†).[3] Confocal microscopy (200 nm slices) of the fluorescein labelled micro-doughnuts revealed that labelling was throughout the structure (Fig. 4, see ESI for movie†), indicating full chemical accessibility and stability of the doughnuts to solvents such as dimethylformamide. Confocal microscopy confirmed that the 1 μm "hole" transversed the doughnut and was not merely a surface defect or dimple. Fluorescein labelled micro-doughnuts were incubated with a variety of mammalian cells (mouse fibroblast (L929), mouse macrophage (RAW264), human embryonic kidney (HEK293T), human ovarian cancer (HeLa), mouse melanoma (B16F10), erythroleukemic (K562) and mES (E14)) at 37 °C/5% CO_2 for 6, 12 and 24 h at a concentration of 86 μg mL^{-1}. Uptake was quantified by flow cytometry using 0.4% trypan blue in Hank's Balanced Salt Solution (HBSS) which quenches any extra-cellular fluorescence of cell surface bound particles,[17] thus ensuring that only those particles that enter the cells are detected. Internalisation was confirmed by pseudo-confocal microscopy after the cells were fixed in 3% paraformaldehyde and stained with Alexa Fluor® 568-phalloidin and Hoechst 33 342 (Fig. 5). Cellular appetite for the doughnuts could be controlled by alteration of the incubation time and doughnut concentration and was typically limited to one or two per cell.

Although the analogous beads (Fig. 1) showed little discrimination in terms of cellular uptake, the doughnuts were much more selective (Table 1). Moreover, discrimination was demonstrated in primary human lymphocytes where minimal uptake was observed for doughnuts. To illustrate this discrimination, HEK293T cells were "beadfected" with Cy5 labelled microspheres and B16F10 cells with dansyl labelled microspheres; they were then mixed and re-grown over 24 h. Rhodamine B labelled micro-doughnuts were added to mixed cell cultures and after 24 h flow cytometry was performed to ascertain which cell line contained micro-doughnuts. Overwhelmingly, HEK293T cells preferentially took up micro-doughnuts over B16F10 cells, a result confirmed by microscopy (Fig. 6). We believe that it is the unusual doughnut morphology that results in cell specificity rather than their size as previous work with 5 μm microspheres showed little cell specificity (unpublished data).

In order to demonstrate the applicability of these types of materials, their *in vivo* fate was determined after intravenous injection (tail vein injection, 30 μg) into a six week old mouse (all animal experiments were undertaken with an approved licence from the Animal Scientific Procedure Division of the Home Office, London, UK). After 4 h, the doughnuts were detected solely in the liver region (Fig. 7) and not in other organs with histology showing that the doughnuts were within the liver parenchyma (Fig. 7). In addition, no adverse effects were observed in the animal following injection.

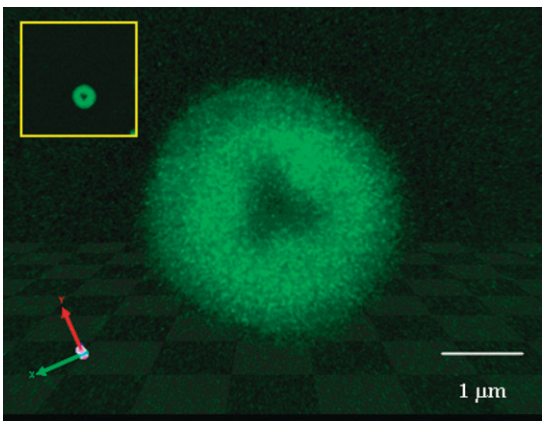

Fig. 4 Confocal microscopy image (Leica DM IRE2) of a fluorescein labelled doughnut.

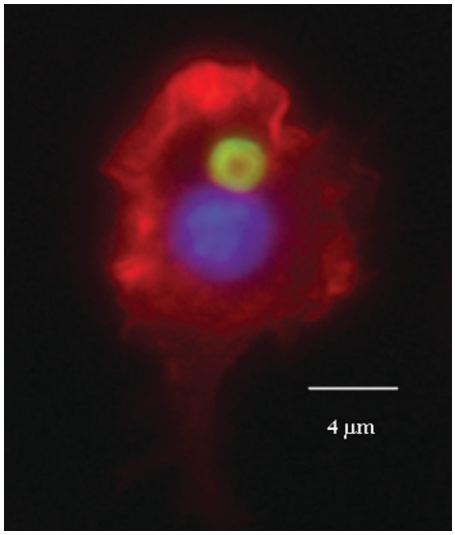

Fig. 5 A single fluorescein labelled doughnut within a RAW264 cell (nuclei labelled with Hoechst 33342, actin filaments labelled with Alexa Fluor® 568-phalloidin. Images recorded on a Zeiss Axiovert 200M).

Table 1 Analysis of uptake of fluorescently labelled 2 μm beads and doughnuts after 24 h (uptake is the % of labelled cells from the total population using untreated cells as the control (0% uptake))

Cell line	% Uptake for beads	% Uptake for doughnuts
RAW264[a]	80 ± 6.6%	94 ± 3.0%
HEK293T[a]	90 ± 0.8%	50 ± 1.7%
L929[a]	46 ± 0.8%	33 ± 1.3%
HeLa[a]	89 ± 2.1%	18 ± 2.1%
B16F10[a]	75 ± 6.9%	13 ± 2.9%
E14 mES cells[a]	37 ± 0.5%	13 ± 1.8%
K562[a]	42 ± 3.4%	3 ± 0.4%
Primary Human Lymphocytes[b]	52 ± 2.0%	0.2 ± 0.04%

[a] Duplicate experiments and $n = 2$. [b] Triplicate experiments and $n = 2$.

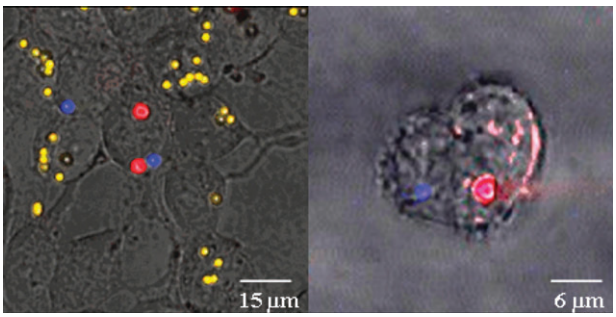

Fig. 6 Left: B16F10 cells beadfected with dansyl microspheres (yellow) and HEK293T cells "particle-fected" with Cy5 beads (blue) and rhodamine B doughnuts (red). Right: HEK293T cell co-labelled with a Cy5 bead (blue) and rhodamine B doughnut (red).

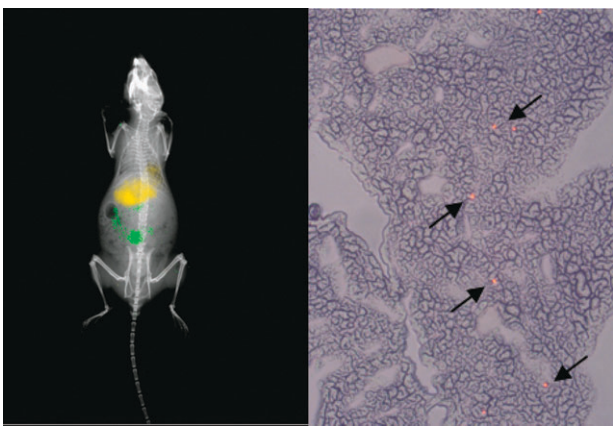

Fig. 7 Left: Optical whole body image (Kodak FX-PRO Reflectance System using multi-spectral un-mixing software) of Cy7 labelled doughnuts (yellow) in the liver. Food (chlorophyll – green) is shown in the gut. Right: Histology on cryostat sections of the liver showing rhodamine B doughnuts (red, black arrows) in the liver parenchyma (imaged using a Zeiss Axiovert S100 inverted microscope).

In conclusion, polymeric composites, with an unusual structure, were produced *via* the dispersion polymerization of styrene in 5% dioxane in ethanol, with accessible amino functionality distributed throughout the doughnut matrix. Their selective cellular uptake has been demonstrated in a variety of cell lines (including primary human lymphocytes) and when in competition, their selectivity for one cell line over another is clear. Additionally, they do not display any toxic effects whilst specifically targeting the liver *in vivo*. These particles may have applications in cellular separations, as selective cell tags or in site-specific liver disease therapies.

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Notes and references

‡ Particles were prepared following standard procedures[3,5,6] with styrene (97 mol%) in the presence of divinylbenzene (DVB, 1 mol%), vinylbenzylamine·HCl (VBAH, 1 mol%) and the initiator (AIBN, 0.01 equiv.) in ethanol.

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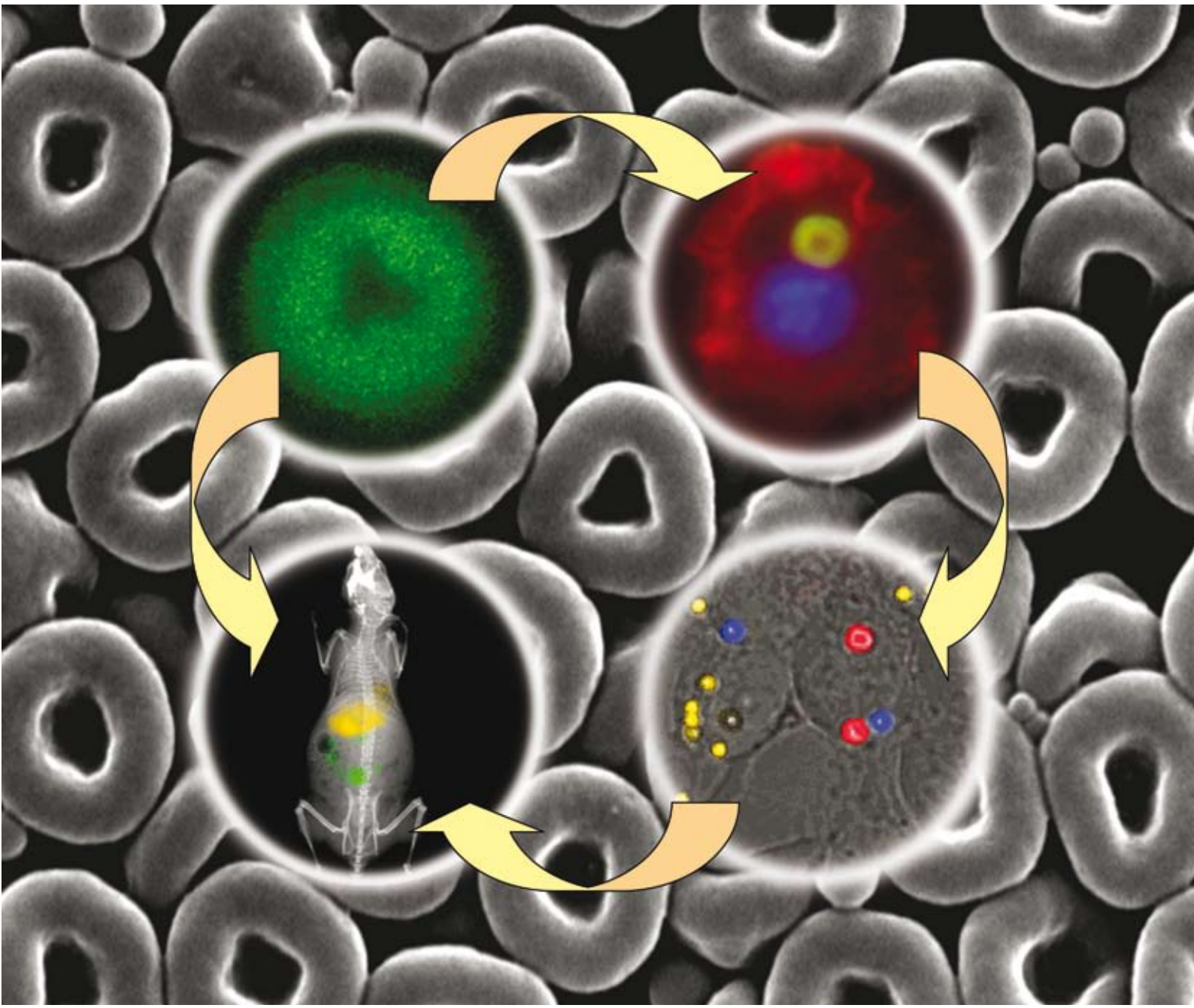

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