We report the synthesis of two simple copolymers that induce rapid cell aggregation within minutes in a fully reversible manner. The polymers can act as self-supporting “cellular glues” or as “drivers” of 3D cell spheroids/aggregates formation at minute concentrations.

The interaction of cells with the outer environment is primarily governed by numerous signalling cascades occurring on the cell membrane via ligand-receptor interactions, which determine vital processes of the life-cycle of cells including differentiation, migration, mitosis, and apoptosis signalling. Hence, the cell membrane is arguably the most important cellular organelle to probe and direct specific cellular functions for therapeutic and research purposes.

Cell surface remodelling (CSR) has emerged as a powerful approach to control numerous biological (and often unnatural) functionalities of cells including protection from the immune system, cryo-preservation, cell immobilization and encapsulation, biopatterning, receptor targeting, as well as three-dimensional (3D) microtissue fabrication and organ transplantation. In recent years, various (bio-)chemical approaches have been reported to functionalize and/or derivatize the cell membrane with various components including nucleic acids, peptides, synthetic polymers and nanoparticles for cell therapy. CSR is particularly useful in the construction of 3D cell/tissue spheroids, and cell-biomaterial ensembles for tissue engineering applications as the concentration of the synthetic component, i.e. the biomaterial, is minute and hence the possible cytotoxic or immune responses are minimised. Current cell aggregation methods via CSR include direct cell membrane biotinylation, covalent crosslinking, and polyelectrolyte or ionic mediated cell aggregation. The further development of such biomaterial-cellular ensembles at the nanoscale is of paramount importance in order to mimic the mechanical, biochemical and interaction cues that occur in the physiological setting.

In the present work, we report on two simple cell membrane interacting polymers that induce and control cell aggregation cascades in a fully reversible manner (Fig. 1).

Polymer P1 was synthesized by free radical polymerization (FRP) of N-vinylpyrrolidone (NVP) acting as a water-soluble element, and 3-(acrylamido)phenylboronic acid (APBA) as a cis-diol reacting moiety targeting cell membrane glycoprotein residues via covalent (but reversible) boronate ester bonds. The $M_n$ of P1 was determined by size exclusion chromatography (SEC) to be 14100 Da; $^1$H nuclear magnetic resonance (NMR) was used to determine the relative monomer feed composition of the two monomers on the final polymer, which was found to be 99.5:0.5 (NVP:APBA) (ESI, Table S1 and Fig. S1).

Polymer P2, also synthesized by FRP, is a copolymer of dl(ethylene glycol) methyl ether methacrylate (DEGMA), which acts as a thermoresponsive element, and N-hydroxysuccinimide methacrylate (NHS-MA), a targeting motif of free primary amino groups on membrane proteins via covalent amide bond formation. The $M_n$ of P2 was found to be 29800 Da by SEC; the ratio of the two monomers on the final polymer was determined by $^1$H NMR (Table S1 and Fig. S2) and was found to be 99:1 (DEGMA:NHS-MA). P2 had a lower critical solution temperature (LCST) onset at sub-cell culture conditions (ca. 14°C) to enable rapid cell aggregation formation even at room temperature.

Both copolymers were found to induce rapid cell aggregation within minutes at relatively low concentrations in complete culture media that was fully reversible, albeit with distinct de-aggregation mechanisms. Upon mixing with human dermal fibroblasts (HDF), P1 triggered rapid formation of cell aggregates at relatively low concentrations (200 μg/mL) due to the inter-cellular crosslinking of neighbouring cells via diol-boronate ester formation with cell surface glycoproteins (e.g. sialic acid rich moieties), as shown in Fig. 2(a) and movieS1 (ESI). It should be noted that although the optimum pH for the
formation of diol-boronate esters is close to 8 (the pKa of boronic acid is 8.6\(^3\)), at physiological conditions, there is still sufficient amount of ionisable boronic acid groups (ca. 6% of the total boronate moieties\(^3\)) that bind to cis-diols present on the cell membrane at pH 7.4. One approach to further improve the rate of boronate ester formation would be the introduction of cationic groups on the polymer backbone in close proximity with the boronic acid to reduce the optimum pH close to the physiological.\(^3\) Although this could be a promising approach, the cationic nature of the polymer would trigger coulombic attraction of the polymer and the anionic cell membrane, and hence the specificity of the system would be potentially compromise.

In order to probe the specificity of the interaction of the polymer-bound boronic acid with cell membrane diol-rich carbohydrate residues, a competition assay was performed by addition of increasing amounts of glucose in the culture medium (Fig. 3(a)). It was observed that a gradual increase of the free glucose concentration resulted in a gradual reduction of the average size of the cell aggregates up to a critical point above which no aggregates could be observed (at 0.01 mM glucose). Additional control experiments with APBA-free polymers were conducted where no cell aggregates were observed under the same experimental conditions (Fig. S5(a)).

In a similar procedure, P2 was rapidly installed on the cell membrane via amide coupling with free amino groups of membrane proteins\(^11\) by simple mixing with the cells in complete medium. Intercellular-type aggregation was quickly observed owing to the hydrophobic interactions of the DEGMA residues above the polymers’ LCST (Fig. 2(b), movieS2 and Fig. S4, ESI). The macroscopic cell aggregation process was found to be fully reversible by simple lowering of the temperature.
below the polymer’s LCST followed by gentle shaking of the cell culture well plate. Interestingly, it was possible to perform a full thermal cycle in order to demonstrate the reversibility of the cell aggregation process without compromising cell viability (Fig. 3(b)). Various control experiments were also conducted to probe the specificity of the coil-to-globule transition of P2 on the cell aggregates formation; a poly(DEGMA) homopolymer (without the NHS residue) was used as a control where, indeed, we observed the absence of cell aggregates under the same experimental conditions (Fig. S5(b)). In a second control experiment, a scramble non-thermoresponsive poly(ethylene glycol) (PEG, Mw, 1500) polymer was used to eliminate the possibility of viscosity-triggered type of cell aggregation that has been observed in erythrocytes cultures upon addition of PEG based polymers.(Fig. S5(c)). Therefore, it is concluded that the mechanism of cell aggregation is derived from the covalent anchoring of P2 on the cell membrane concerted by temperature modulated coil-to-globule polymer transition that macroscopically drives the cell aggregation process.

In order to demonstrate the generic nature of the proposed aggregation mechanisms for both polymers, we performed the same sets of experiments on a model lung cancer (A549) cell line. Again, both polymers induced rapid aggregates formation at similar rates as observed in the case of the HDF cell line (Fig. S6).

Finally, to visually monitor the presence of the polymers on the cell membrane, we synthesized fluorescent polymer derivatives of P1 and P2 by incorporating fluorescein methacrylate tags on the polymers’ backbone (see ESI). The cells appeared with a characteristic green fluorescent shell in the non-adherent state upon mixing with either P1 or P2, proving the direct interaction of the polymers with the cells’ membrane (Fig. 4(a) and (b)).

We then sought to quantify the rate of the cell aggregates formation for P1 and P2 with time; the cell aggregates were found to increase in a non-linear rate with time, with relatively fast rates of aggregation for both polymers in the first 20 minutes followed by lower rates until the aggregates were completed at about 60 minutes (Fig. 4(c)). This result is further corroborated by the time-lapsed images (Fig. S4, Fig. S6 and movieS1, ESI) where it is observed that the aggregates are formed by the initial growth of small “cell islands” consisting of a few cells which aggregate together to form larger clusters.

Furthermore, no toxicity was observed for both polymers in HDF after 24 and 48 hours period even at high polymer concentrations (up to 500 μg/mL) (Fig. 4 and Fig. S7). P1 was found to be non-toxic in A549 cells, whereas P2 demonstrated significant, but acceptable cytotoxicity on these cells. The live/dead fluorescence assay supported this observation with the majority of cells remaining alive after 48 hours incubation period (Fig. S7).
biomaterials\textsuperscript{37-39} for tissue regeneration or the formation of millimetre sized 3D tumouroids\textsuperscript{40, 41} for \textit{in vitro} modelling applications. Strikingly, both copolymers could form macroscopic cell-laden gels under physiological conditions observable by the naked eye that could be reversibly turned to their corresponding sol state either by the addition of glucose (in the case of P1) or lowering the temperature (in the case of P2), as illustrated in Fig. 5. The gels could remain stable for hours without any observable change or any significant cytotoxicity on either of the cell lines tested. Rheological studies further corroborated the sol-gel type transition of both copolymers controlled by their corresponding stimuli (Fig. 58).

In conclusion, we have synthesized simple copolymers that induce rapid cell aggregation in a fully reversible manner at minute concentrations in complete culture medium. We anticipate that these polymers will find uses as injectable “cellular glues” for \textit{in vivo} tissue regeneration and cell transplantation or in the construction of 3D cell spheroid/tumoroid models.

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Notes and references

1. B. M. Gumbiner, Cell, 1996, 84, 345-357.