Freely Suspended Cellular “Backpacks” Lead to Cell Aggregate Self-Assembly

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Freely Suspended Cellular “Backpacks” Lead to Cell Aggregate Self-Assembly

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Cellular “backpacks” are a new type of anisotropic, nanoscale thickness microparticle that may be attached to the surface of living cells creating a “bio-hybrid” material. Previous work has shown that these backpacks do not impair cell viability or native functions such as migration in a B and T cell line, respectively. In the current work, we show that backpacks, when added to a cell suspension, assemble cells into aggregates of reproducible size. We investigate the efficiency of backpack–cell binding using flow cytometry and laser diffraction, examine the influence of backpack diameter on aggregate size, and show that even when cell–backpack complexes are forced through small pores, backpacks are not removed from the surfaces of cells.

Introduction

There exists a new, burgeoning field of biohybrid materials in which synthetic materials are functionally integrated with cellular species while leveraging both biological and material properties and behaviors. Synthetic materials systems such as anisotropic microparticles,1 muscular thin films,2 thermally responsive films with integrin ligands,3 films capable of sensing and selectively releasing dead cells,3 magnetic micromanipulators,5 nanoparticulate cellular patches,6 and functional cell backpacks7 have recently been reported offering exciting possibilities for a new class of biomaterials based on the symbiosis between synthetic building blocks and native biological behavior.

Cellular “backpacks” are nanoscale thickness, micrometer-sized, photolithographically patterned heterostructured multilayer systems capable of noncytotoxically attaching to the membrane of a living cell.7 Cellular backpacks have been attached to the surface of two types of living immune cells without impairing their native behaviors. If a backpack is attached to a cell that normally performs a useful function, such as homing to solid tumors or areas of trauma, then these native behaviors can be leveraged to deliver functional materials contained within the backpack. Diagnostic (such as imaging) or therapeutic (such as delivery) payloads are possible, as well as combining several modalities in a single platform.

Each backpack contains a functional payload which may be any material that can be integrated in multilayer or homopolymer thin films, including drugs, imaging contrast agents, and nanoparticles. The attachment mechanism between the backpack and the cell surface must be chosen based on the cell type of interest. In this work, we used a B cell line that expresses an abundance of the cell surface receptor CD44, for which the natural ligand is hyaluronic acid (HA). One face of the backpack consists of a HA-containing multilayer that attaches to the membrane of one or more cells.

In previous work,7 backpacks were fabricated on a glass slide and tethered to the substrate via a pH- and temperature-labile region. Cells were attached to backpacks at a controlled ratio (R = No. of cells/No. of backpacks) via the CD44-HA interaction and released upon lowering the temperature. This yielded cell–backpack complexes with a well-defined number of cells and backpacks. While this technique afforded great control over cell–backpack association, the effort-intensive process of seeding and releasing on a 2D surface may limit its clinical relevancy. An alternative method is one where the backpacks are released and collected from the fabrication substrate ex vivo and exposed to cell suspensions. We refer to this approach as an injectable formulation because backpack solutions could easily be loaded into a syringe and injected into a patient. Because injectable backpacks are free to attach to cells in many different configurations, including multiple cells per backpack and vice versa, cell–backpack aggregates form.

Suematsu et al.8 recently reported forming immune cell aggregates for tissue engineering applications. A collagen scaffold seeded with stromal cells was transplanted into mice. This traditional tissue engineering approach produced artificial lymphoid-like organoids that functioned much like secondary lymphoid organs, recruiting B and T cells and forming follicular dendritic cell networks. This work offers exciting possibilities in engineering hybrid synthetic-biological devices for treating immunodeficiency diseases. Their approach, however, requires surgical implantation techniques for introducing the lymphoid-like tissue into the body, and much greater clinical ease may be found in methods able to form organoids by simply injecting a cell-containing suspension.

Cellular backpacks may offer an alternative strategy to create injectable synthetic lymphoid organoids that achieve the extremely high cell density typical of lymphoid tissues. Cells could be mixed with backpacks to form aggregates that may be passed through small pores (for example, a needle tip), disaggregate, and dynamically reform. Because the backpacks do not occlude the entire cell surface, cells are free to interact with the environment, an essential requirement for immune system components. Motivated by the work of Suematsu et al. and our
original observation that cells would aggregate upon freely suspended backpack exposure, we sought to create cellular aggregates that are reversibly associated but with enough cell—backpack association strength to withstand mechanical challenges.

In this paper, we present fundamental studies on forming cellular aggregates using injectable cellular backpacks, how to control aggregate size, and observations on cell—backpack association strength. We found that two parameters strongly determined the size and character of aggregates: the ratio of cells to backpacks in a culture and the diameter of the backpack. Using confocal microscopy, flow cytometry, and laser diffractometry, we observed that, while very large (>1 mm) aggregates can form, aggregates may also dissociate and reform. Aggregates were forced through a nylon mesh filter and observed afterward: as the filter size decreased, resulting aggregates were smaller. For a pore size less than the diameter of the cell, backpacks were still attached, perhaps indicating a sufficiently strong cell—backpack association required for a backpack to remain attached to a lymphocyte undergoing extravasation in vivo. We feel that an injectable backpack system could have applications in lymphoid tissue engineering as described by Suematsu,6 as well as more general cellular engineering applications requiring close cell association.

Materials and Methods

Materials. Poly(methacrylic acid) (PMAA, PolySciences, M = 100 kDa), poly(vinylpyrrolidone) (PVON, Aldrich, M = 1.3 MDa), poly(diallyldimethylammonium chloride) (PDAC, Aldrich, kDa in 20% aqueous solution), poly(styrene sulfonate) (SPS, Aldrich, M kDa), poly(vinylpyrrolidone) (PVPON, Aldrich, Sigma, DS), poly(lactide-glycolide) (PLGA, Sigma, Ms = 5–15 kDa) were used without purification. Cells were passed and maintained in RPMI with l-glutamine (Mediatech), penicillin/streptomycin (P/S, Mediatech), and fetal calf serum (characterized FCS, Mediatech). 3.2-Dicarboxylic-acarbocyanine perchlorate (DiO, Molecular Probes), which fluoresces at the same wavelengths as fluorescein isothiocyanate (FITC), was used to stain the PLGA backpack. Iron oxide magnetic nanoparticles (MNP, Fe3O4, 10 nm diameter, Ferrotec EMG 705) stabilized with an anionic surfactant were used. Hank’s Balanced Salt Solution (Gibco) was used to wash cells, and propidium iodine (PI, Calbiochem) was used as a viability dye. Additional information on solution concentrations and pH conditions used during depositions can be found in the Supporting Information.

Backpack Fabrication. We used a previously described7 aqueous-based layer-by-layer technique to deposit the polymer films. One significant exception is the PLGA region of the backpack in the current study, which was assembled using a spray technique. A solution of PLGA (1 mg/mL) and DiO (1 mg/mL) in chloroform was sprayed (10 mL/min for 30 s) onto the surface of a (PMAA2.0/PVPON2.0)20.5 multi-well slide, which was then coated using a Badger 105 air brush powered at 0.33, and 0.5 mL aliquots were passed through 25 mm diameter nylon mesh filters of three different opening sizes (20, 30, and 60 µm; Millipore) using a reusable syringe filter (Pall). These aliquots were transferred to 15 mm tubes and chilled on ice.

Flow Cytometry and Confocal Microscopy. Aliquots of cell—backpack complexes were analyzed on a BD FACS Canto II flow cytometer. The cell viability marker propidium iodide (PI, 50 µg/mL PBS) was added during backpack attachment at 40 µL/106 cells (i.e., a final 2 µg/mL PI concentration with 106 cells/mL). Data sets of 105 events were collected following agitation. Data analysis was performed to 18 mL pH 7.4 PBS in a quartz cuvette. Data were collected before and after gentle agitation using the built-in magnetic stir bar. All data shown were collected following agitation. Data analysis was performed using a FlowJo software.12 which does not require the input of a refractive index.

Nylon Mesh Filtering. Backpacks were attached to cells at R = 0.33, and 0.5 mL aliquots were passed through 25 mm diameter nylon mesh filters of three different opening sizes (20, 30, and 60 µm; Millipore) using a reusable syringe filter (Pall). These aliquots were placed in 4 well LabTek chambers and observed using confocal microscopy.

Results and Discussion

Backpacks were assembled on a glass substrate using a photolithographic lift-off technique.13,14 Photore sist was deposited and patterned with 7 or 15 µm circles on a (PDAC4/SPS15.5)-coated glass slide, which was then coated using a...
backpack above pH 6.2, as shown previously,20 this critical dissolution pH is due to the deprotonation of PMAA carboxylic acids, which are participating in hydrogen bonds. When this mechanism is used, backpacks can be released from the fabrication substrate and collected, then attached to cells in an ex vivo cell culture or injected directly into the body where cells of interest may bind to specific ligands on the backpack surface.

An “injectable” backpack formulation, however, leads to cell—backpack aggregates. These aggregates contain any number of cells and backpacks, and the factors influencing the order of these aggregates include the number of cells per backpack and the number of backpacks attached to each cell. Nonconformal attachment can occur due to curvature of the flexible backpack; an overhanging portion of the backpack may then bind to one or more cells. An example is shown in Figure 2a, where three cells attached to a single backpack. When a single cell is associated with more than one backpack, and each backpack may attach multiple cells, aggregates form. Figure 2b shows one of the lowest order aggregates that may form, where one cell has two backpacks and each backpack has three cells attached. Each of these micrographs shows cells exposed to backpacks for ∼1.5 h, and no evidence of internalization was ever observed. (In fact, when backpacks are exposed to a macrophage cell line known to quickly internalize spherical particles several micrometers in diameter, we see very little backpack internalization. This is the subject of ongoing investigation and will be featured in an upcoming publication.)

As will be shown later, aggregate size depends on (1) the number of cells associated per backpack and (2) the number of backpacks per cell. Backpack size, controllable during fabrication, will strongly influence the number of cells associated per backpack7 (see Figure 2a,b). We fabricated backpacks of two different diameters (d = 7 and 15 µm) and controlled the number of backpacks associated per cell by changing the ratio of cells to backpacks (R = No. of cells/No. of backpacks). We find that aggregate size monotonically decreases with R and increases with d (for a given R).

Figure 3 shows flow cytometry plots and confocal micrographs of cell—backpack (d = 7 µm) aliquots for R = 1—0.1 (for easier visualization, similar plots and micrographs for R = 10—3 may be found in the Supporting Information). Shown are FITC signal versus forward scatter (FSC) data from flow cytometry: cell aggregates are detected at higher FSC, and
aggregates associated with one or more backpacks are detected at higher FITC values (since each backpack contains DIO in the PLGA region, which fluoresces almost identically to FITC). Thus, aggregates with backpacks are found in the upper right quadrant, and single cells with one or more backpacks are found in the upper left quadrant. We used confocal microscopy to directly observe aggregate size, which dramatically increases with decreasing $R$. For $R > 1$, we see very small aggregates (less than three cells), with primarily only one backpack associated per cell (see Supporting Information for confocal micrographs and flow cytometry plots). At $R = 1$, larger aggregates begin to form, and by $R = 0.2$, large complexes are found. At $R = 0.1$, a single aggregate formed in the dish; the micrograph in Figure 3 shows only the edge of this aggregate. To further quantify these aggregate structures, flow cytometry analysis of backpack fluorescence versus FSC on cell−backpack aliquots shows that as $R$ decreases, the number of cells associated with a backpack increases. Because the flow cytometer passes the cell suspension through a small quartz capillary, aggregates break up before passing through the laser path for analysis. This limits analysis to small aggregates, single cells, and single backpacks (which are excluded from this analysis based on PI signal and FSC value), though the starting aliquot included large aggregates. As laser diffraction data indicates, the large aggregates seen in the optical images below are associated via both strong, specific, CD44-HA interactions and weak, nonspecific, cell−backpack binding. Small clusters, as seen in Figure 2, associate only via the strong CD44-HA interactions, and these are the FSC$^\text{high}$ events shown in Figures 3 and 4. A detailed discussion of how different association strengths lead to large aggregates versus small cell clusters is presented along with the laser diffraction data below.

The backpack diameter $d$ also strongly influences the size of aggregates. Figure 4 shows confocal images and flow cytometry plots of cell−backpack aggregates formed with $d = 15 \mu m$ backpacks. Aggregate size trends are similar to the $d = 7 \mu m$ case, but the onset $R$ value at which aggregation begins increases to greater than $R = 10$. Indeed, the aggregates seen for $d = 7 \mu m$ and $R = 0.33$ are roughly the same size as those seen for $d = 15 \mu m$ and $R = 10$. This suggests a superposition of the $d$ (backpack diameter) and $R$ (number of backpacks per cell) variables.

As can be seen in Figure 3, FITC$^\text{high}$ events have two distinct populations differing by a factor of 2 in FSC intensity. This reflects single cells with a backpack or small aggregates with one or more backpacks associated. Figure 5 shows the percent-
age of FITC_{high}/FSC_{low} (single cells with a backpack) and FITC_{high}/FSC_{high} (small clusters) events, as well as the sum, for both $d = 7$ and $15 \mu m$. For $d = 7 \mu m$, at $R = 10$, $3\%$ of cells are associated with a backpack; at $R = 0.1$, $65\%$ of events include a backpack. When the diameter increases to $15 \mu m$, the highest number of cells with an attached backpack decreases to $46\%$. While this might reflect slight differences in sample handling, it is more likely that this decrease is due to curling of some backpacks upon themselves, thus, reducing the total surface area available to strongly bind. Examples of how $d = 15 \mu m$ backpacks fold are seen in Figure 6. This curling behavior was not observed for $d = 7 \mu m$ backpacks, suggesting some critical size required for folding.

As cells pass through the cytometer's fluidics system, the solution is forced through a small capillary. The values reported in Figure 5 are lower bound estimates for the true number of cells associated with backpacks, because some backpacks will be sheared off the surface of cells during flow through the instrument.

We used laser diffraction to further quantify the nature of these aggregates and investigate their association strength. Aliquots of cell—backpack complexes mixed at the same ratios as above show increasingly large aggregates with decreasing $R$, which agrees with the confocal microscopy results presented above. Unlike the confocal results, all diffraction samples were mildly agitated (at about 100 rpm using a built-in stir bar) before analysis. Prior to agitation, most samples show an extremely large aggregate distribution curve (mean > 1 mm) that is not constant with time; very large fluctuations led to inconsistent data. Upon agitation, this distribution falls to the curves shown in Figure 7, which are consistent and reproducible. Furthermore, if agitation was stopped, the large aggregate distribution appeared again, showing that aggregate dissociation is reversible. An agitation-dependent distribution for $R = 0.33$ is provided in the Supporting Information, as well as confocal microscopy images of before- and after-agitation aliquots.

In Figure 7, B cells are shown as the dashed line, which has a distribution mean of $\sim 15 \mu m$, slightly smaller than the $17 \mu m$ backpacks. These curves show two populations, one centered at $\sim 15 \mu m$ (single CH27 cells) and the other at an increasingly greater diameter, depending on $R$. For $d = 7 \mu m$ backpacks, a clear second peak appears at $R = 1$; this second peak appears at $R = 3$ for $d = 15 \mu m$ backpacks. Individual, nonbackpacked CH27 cells are shown as the dashed line.
μm cell diameter observed by microscopy. At $R = 10$, we see a similarly shaped curve shifted to the right, suggesting one-backpack-to-cell complexes. As $R$ decreases, multicell, multi-backpack aggregates begin to form, both shifting the mean value higher and changing the shape of the curve to include a broad shoulder. At $R = 1$ for $d = 7 \mu m$ and $R = 3$ for $d = 15 \mu m$, a second peak emerges, indicating a distinct aggregate population. Consistent with the confocal results above, as $d$ increases the aggregation-onset $R$ value increases as well.

From the flow cytometry and laser diffraction data, we find that aggregates are able to dissociate into smaller cell-backpack clusters. The number of cells in each cluster depends on $R$ and $d$, and these clusters weakly bind together to form the large aggregates seen in Figures 3 and 4. Additionally, this association–dissociation event is reversible; once agitation is stopped, very large aggregates were observed again. The association in a small cell cluster is based on CD44-HA interactions between the membrane and the HA-containing cell-adhesive region. Binding between clusters to form large aggregates are much weaker and is likely based on nonselective interactions between cells and the outer face of the backpack (which contains some or all of the hydrogen-bonded release region). These nonselective interactions are weak enough to be compromised with even mild agitation. Binding interactions in the small cell clusters, however, are not compromised by even very harsh agitation (maximum stir bar rotation).

To further test how strongly backpacks are attached to B cells in clusters and aggregates, aliquots of cells and backpacks ($R = 0.33, d = 7 \mu m$) were collected and passed through nylon mesh filters of varying aperture size. This experiment provides a rough approximation of extravasation, the process by which immune system cells leave the circulatory system and enter tissue. This process requires the cells to squeeze through very tightly apposed endothelial cells,24 exerting shear forces on the surface and challenging the adherence of any attached object.

The average diameter of a B cell is $\sim 17 \mu m$, and four mesh sizes were chosen to challenge the aggregate association strength as well as the cell–backpack interaction. Figure 8 shows that, for mesh opening sizes of 11, 20, 30, and 60 μm, a significant number of cell–backpack complexes remain after filtering. Consistent with the agitation-dependent, reversible aggregation behavior seen above, aggregates are dissociating into smaller aggregates or cell clusters (i.e., cells attached to a backpack via strong CD44-HA interactions) while passing through the mesh. (The total number of cells in the filtrate is comparable to the prefiltered aliquot, indicating that very few clusters or aggregates are actually removed during filtering.) After this dissociation, small aggregates and clusters are then free to reform larger aggregates. The size of the remade aggregates decreases with decreasing mesh size, since the original large aggregate is broken down into smaller clusters or aggregates. This demonstrates that the size of the temporary small aggregate or cluster created immediately after filtering influences the final remade aggregate size.

The 11 μm pore size case is of particular interest because it is less than the average diameter of a B cell. Clusters in the filtrate are very small; primarily, cells are associated with only one backpack. This result suggests that even though a cell was forced to deform as it passed through the pore, the backpack remained on the surface. While this does not directly correlate to the active, receptor-mediated process of extravasation,24 it does suggest that the cell–backpack association is sufficient to resist moderately strong mechanical challenges.

Conclusions

Cellular backpacks may be used to create aggregates of a model B-lymphocyte cell line. Two variables were examined to affect the size of the aggregates: the ratio of cells to backpacks, and the backpack diameter. By decreasing the ratio $R$ of cells to backpacks, we increase the size of the aggregate. As the diameter of the backpack increases (for the same $R$), so does the aggregate size. Flow cytometry results indicate that for $R = 0.1$ and $d = 7 \mu m$, greater than 65% of cells will be associated with a backpack. When $d$ increases to 15 μm at $R = 0.1$, greater than 45% of cells will remain attached to a backpack. When aggregates formed with $d = 7 \mu m$ backpacks are forced through a mesh filter for varying pore sizes, aggregates will dissociate and reassociate. As the pore size decreases, the final aggregate size decreases as well. For the smallest pore size, 11 μm, backpacks remain associated with cells even though this is less than the diameter of the cell, suggesting a strong interaction between the cell and backpack.

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Supporting Information Available. Details on photolithographic fabrication, flow cytometry, and confocal microscopy for $R = 10$–3 for $d = 7 \mu m$ backpacks and laser diffraction data showing the agitation-dependent dissociation of aggregates. This material is available free of charge via the Internet at http://pubs.acs.org.
References and Notes


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