康宁可溶性微载体产品手册

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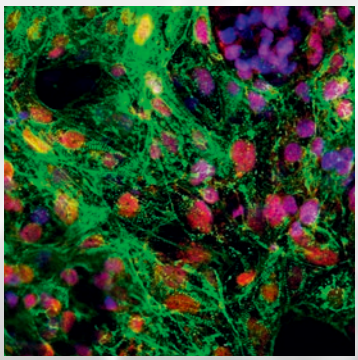

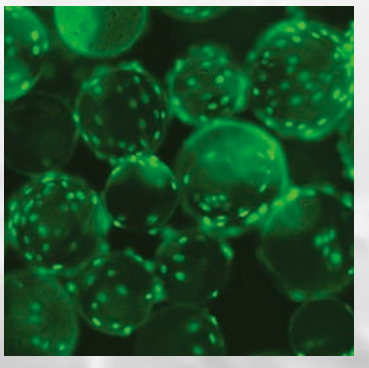

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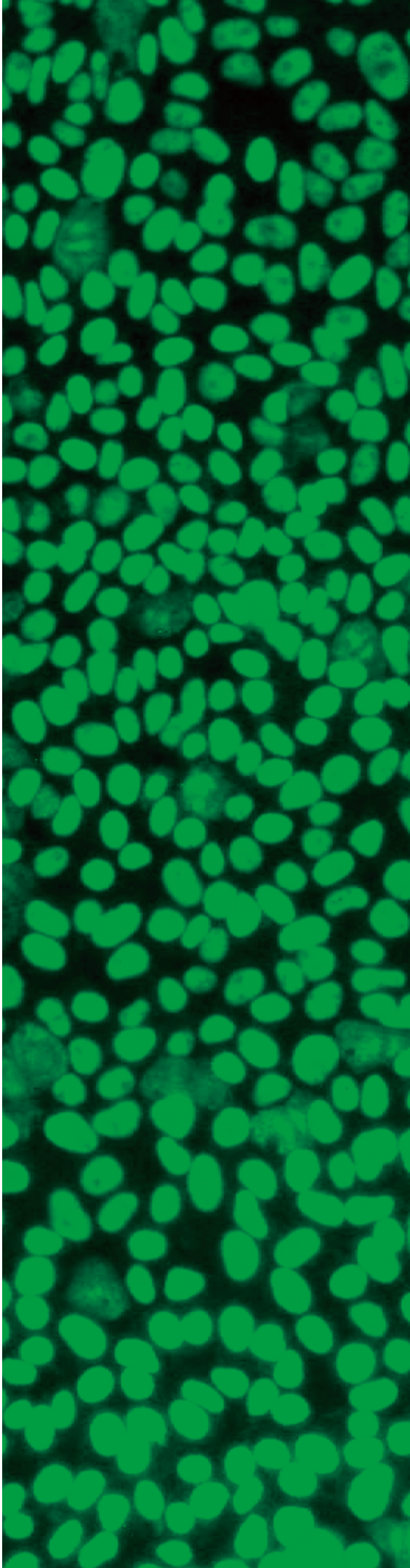


新一代微载体推动细胞治疗进程

微载体是在搅拌培养中支持细胞贴壁和生长的直径 100-300 µm 的小球。它通过降低大规模生产中的固定成本（如场所、人力资源）和可变成本（如培养基消耗）来实现更高效的细胞生产。

选择最适合的微载体需要考虑的关键因素是产品的预期用途。对于细胞治疗而言，其产品是活的具有功能的细胞。然而，市面上大多数微载体具有不适用于细胞治疗应用的显著缺点。它们由固定的基质构成，这就要求下游工艺需要将微载体与消化下来的细胞分离。这个分离步骤是毫无价值的，增加了生产过程的复杂性和成本。

使用微载体的另一个限制是，细胞扩增后贴壁很牢时，很难有效地从微载体上解离下来。对于这类微载体培养，细胞总产量显著降低，更重要的是，收获细胞的功能可能受到所需的严苛消化方法的影响。

一种创新的解决方案

康宁可溶性微载体为大规模细胞扩增提供了理想的解决方案。可溶性微载体由交联多糖聚合物组成，可在细胞收获期间有效地溶解（图 1）。其能完全溶解的特性带来更简单的下游纯化过程，并且不需要物理分离细胞和微载体。

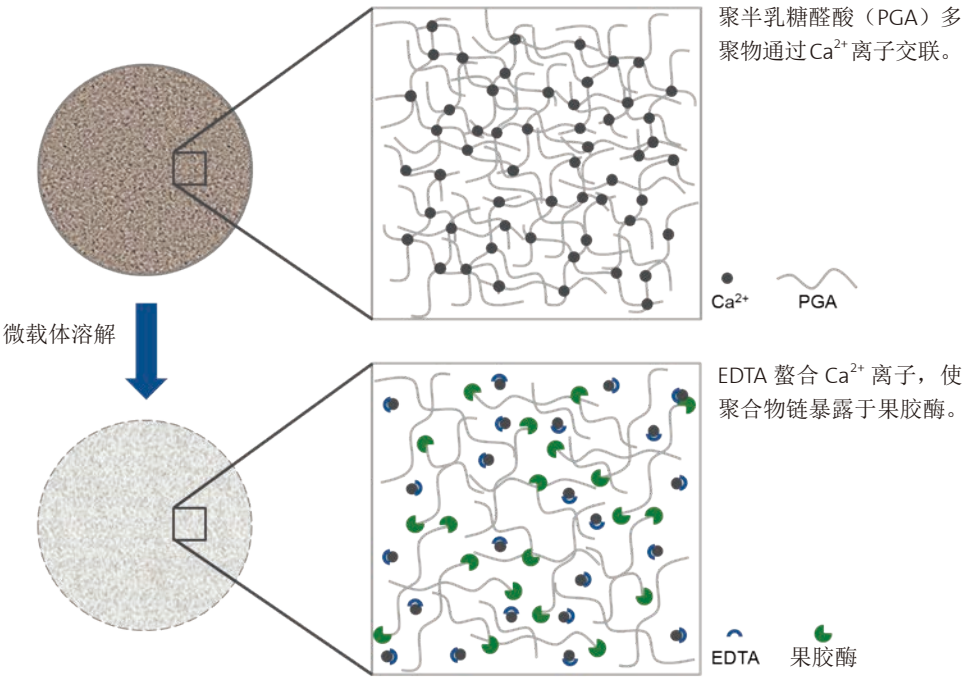


图 1. 在细胞收获时，钙离子交联的果胶酸微载体在 EDTA 和果胶酶的作用下溶解

通过加入含 EDTA、果胶酶和常规细胞消化酶的收获溶液，微载体会迅速溶解。当钙离子被 EDTA 螯合时，微载体聚合物变得不稳定，随后在果胶酶的作用下降解。通过额外的细胞消化蛋白酶（例如胰酶、Thermo Fisher 的 TrypLE 或 Innovative Cell Technologies 的 Accutase），分解细胞 - 细胞相互作用和细胞外基质，最终产生含小分子糖寡聚物的单细胞悬液。

康宁可溶性微载体是由光学透明的、密度 1.01-1.03 g/cm^3、表面积 5,000 cm^2/克、尺寸约 250 µm 的小球组成。与其他市售微载体相比，康宁可溶性微载体尺寸和表面积分布更窄，支持更均匀的细胞接种和生长汇合，从而形成更均一的细胞悬浮液。此外，窄的微载体尺寸范围和密度使得生物反应器内悬浮微载体的搅拌速度更加一致。

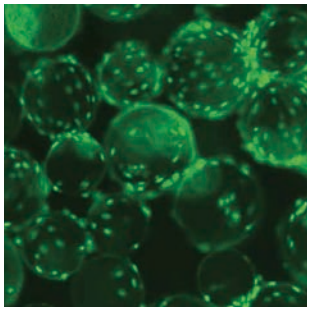
数据支持

一些研究已经证明，使用可溶性微载体代替传统的微载体，可以获得相同或更好的细胞扩增和收获：

- 在无血清培养基中，人间充质干细胞（hMSC）在可溶性微载体上成功地多代扩增（CLS-BP-PST-020，CLS-AN-480）。
- 与聚苯乙烯微载体相比，在可溶性微载体上 hMSC 在更短时间下生长状态相当或更好、细胞收获率更高。该实验在来自不同供体的脂肪和骨髓来源 hMSC 上得到证实（www.corning.com/worldwide/en/products/life-sciences/resources/webinars.html）。
- 可溶性微载体在 5L 生物反应器中支持 hMSC 的扩增和回收。细胞在 20 分钟内从可溶性微载体中回收，并保留标准的狭长形态、正常核型和多能性（CLS-AN-470）。
- 与 Matrigel 包被的聚苯乙烯微载体相比，可溶性微载体上人诱导多能干细胞（hiPSC）的产量增加。与从聚苯乙烯微载体中回收 hiPSC 相比，可溶性微载体支持更简便、温和的细胞回收过程（会议海报和讲座中分享的康宁内部数据）。
- MRC5 和 Vero 细胞在转瓶和生物反应器中可溶性微载体上高效生长和回收（CLS-PST-BP05 和 CLS-BP-PST-020）。

可溶性微载体支持多种细胞类型的高效生产和收获，包括：hMSC、hiPSC、Vero 和 MRC5 等。这种新一代可溶性微载体支持高细胞产量的有效回收，免除微载体分离，从而产生更节省成本和人力的收获过程，并更好地维持细胞健康和功能。

康宁可溶性微载体



康宁新型可溶性微载体，旨在简化细胞收获、分离和浓缩过程。 微载体预灭菌，减少预处理步骤。包被变性胶原蛋白或康宁 Synthemax™ II* 表面，增强细胞贴壁，最大化细胞产量和活力。能在 10-20 分钟内快速分解，不需要分离微载体即可轻松收集细胞。 康宁可溶性微载体使细胞分离比市售微载体更快、更温和、更方便。

适用领域

- 细胞治疗
- 疫苗生产
- 大规模贴壁细胞扩大培养和储存

产品特性

- 可溶解，轻松将细胞与微载体分离
- 透明度好，清晰观察细胞形态
- 无菌保证水平（SAL）10^{-6}
- 预包被，增强细胞贴壁
- 无热原

订购信息

变性胶原包被

货号	产品描述	规格
4979	可溶性微载体，变性胶原包被	1 g/管
4980	可溶性微载体，变性胶原包被	5 g/管
4981	可溶性微载体，变性胶原包被	10 g/管
4982	可溶性微载体，变性胶原包被	100 g/瓶

康宁 Synthemax II 包被 *

货号	产品描述	规格
4987	可溶性微载体，Synthemax II 包被	1 g/管
4988	可溶性微载体，Synthemax II 包被	5 g/管
4989	可溶性微载体，Synthemax II 包被	10 g/管
4990	可溶性微载体，Synthemax II 包被	100 g/瓶

* 康宁 Synthemax II 提供了一种独特的无动物来源的合成表面。Synthemax II 是一种基于玻连蛋白（Vitronectin）的合成多肽，含 RGD 基序及侧翼序列，能支持多种祖细胞及多能干细胞在无血清培养基（如 mTeSR®）中的贴壁和扩增，培养的多能干细胞可分化为多种类型的细胞。

康宁可溶性微载体常见问题解答

1. **可溶性微载体是否有孔？**

   可溶性微载体为水凝胶，能允许水和小分子通过多聚半乳糖醛酸聚合物，但孔径不足以容纳细胞通过。

2. **是否可以用培养基或 Dulbecco's 磷酸盐缓冲液（DPBS）来溶胀可溶性微载体？**

   必须用水来充分溶胀可溶性微载体，其渗透压利于产生尺寸均一的 200-300 µm 直径的微载体。

3. **溶胀的可溶性微载体是球形吗？**

   可溶性微载体不是完美的球体，但溶胀后的可溶性微载体尺寸分布窄，具有均一的表面积。

4. **可溶性微载体溶胀时可以用塑料（聚苯乙烯或聚丙烯）储液瓶代替硅化玻璃瓶吗？**

   可溶性微载体可以在塑料瓶中溶胀，但是微载体可能会粘附在瓶壁上不易被去除，带来微载体的损失。微载体的损失程度取决于储液瓶的表面积和溶胀微载体的量。因此，我们建议使用硅化玻璃瓶以尽量减少微载体的损失。

5. **如果溶胀后在水中观察到漂浮（不能沉降）的可溶性微载体，怎么办？**

   漂浮的微载体可能是由于加水时混入空气所导致。单个微载体含有空气，会使它们浮在液面上。 有两种方法可以去除空气：

   - 轻轻敲击容器的一侧，直到微载体落下并沉降。
   - 重复地拿起容器并用力放到在坚硬表面上。

   所有漂浮微载体的完全沉降可能需要 1 个小时。

   为了防止在溶胀过程中产生漂浮微载体，请缓慢加入足量的水（每克微载体 150 mL），然后再混合。在微载体溶胀期间请朝一个方向旋转搅拌，不要颠倒或剧烈混合。请参考康宁关于可溶性微载体溶胀的技术文档（CLS-AN-467）了解更多详情。

6. **可溶性微载体可以灭菌吗？**

   康宁的可溶性微载体是无菌提供，其无菌保证等级（SAL）为 10^{-6}。 我们不建议高压灭菌可溶性微载体。

7. **可溶性微载体降解后会影响细胞悬液的粘度和渗透压吗？**

   可溶性微载体降解后，粘度和渗透压在标准含血清培养基的预期范围内。注意：粘度和渗透压的变化取决于降解的微载体与收获溶液的比例，如果您优化得到的降解过程是使用小于 250 mL 的收获溶液溶解大于 1 g 的可溶性微载体，您应该预期会有粘度和渗透压的增加。

8. **溶胀后的可溶性微载体可以储存多久？**

   建议在 4℃储存溶胀后的可溶性微载体，最长 1 周。

9. **1 克含有多少可溶性微载体？**

   1 克干燥的产品中约有 2.8×10^{6} 个可溶性微载体。

10. **微载体溶解后，收获液中有哪些成分？**

    采用分子排阻色谱法，我们在收获溶液中观察到半乳糖醛酸短链、果胶酶、EDTA 和蛋白酶。

11. **是否可以降低收获液中试剂的体积或浓度？**

    在多种生物反应器和细胞类型中，每克微载体使用 250 mL 含 100 U/mL 果胶酶和 10 mM EDTA 的收获液，我们均观察到一致的微载体溶解和细胞释放。尽管如此，收获液中果胶酶、EDTA 和蛋白酶的体积和浓度可根据细胞类型和生物反应器的不同而减少。

需主要考虑的三点：

- 在微载体溶解过程中，确保使用足够量的收获溶液使叶轮完全浸没，以提供充分的低剪切力混合。在我们的生物反应器系统中，我们已经在降低收获溶液至 150 mL/g 时实现了溶解。
- 我们已经在果胶酶和 EDTA 浓度分别低至 50 U/mL 和 5 mM 时观察到了微载体的降解。
- 收获溶液体积或浓度的降低可能延长微载体完全溶解并获得单细胞所需的时间。我们建议分别评估这两个变量，再组合，以确定针对您系统的最优化方案。

12. 在可溶性微载体上需接种多大的细胞密度？

建议可溶性微载体的细胞接种密度略高于常规二维平面培养的密度。对于人间充质干细胞，推荐接种密度 4,000-7,500 个细胞 /cm^2，具体取决于培养基类型。

13. 需要使用多大浓度的可溶性微载体？

我们建议在初始评估时使用 5-10 cm^2/mL（1-2 g/L）的微载体浓度。根据搅拌速度、培养基补充时间和细胞接种密度，微载体浓度可以增加到 15-20 cm^2/mL（3-4 g/L）。

14. 可溶性微载体细胞贴壁和扩增过程中可以使用连续搅拌吗？

连续搅拌方案可以用于可溶性微载体细胞贴壁和扩增阶段。但是，我们建议首先在细胞贴壁阶段分别评估静止（在一定时间内不搅拌）、间歇（搅拌 / 不搅拌循环）和连续搅拌方案，以确定哪种方案能更好地实现 > 80％的细胞贴壁以及细胞在所有微载体上均匀分布。在细胞贴壁阶段，采用使微载体悬浮所需的最小搅拌速度。不同的细胞类型、培养基配方（例如有 / 无血清）、微载体表面（如康宁 Synthemax™ II、变性胶原蛋白）和容器类型（如转瓶、生物反应器）的组合需要不同的方案。

15. 由于溶胀后可溶性微载体的透明度高，怎样才能更好地观察溶液中或搅拌培养中的微载体小球？

为了提高溶胀后可溶性微载体的可见度，建议用明亮的手电筒照射溶液。

16. 可溶性微载体是否会在操作处理和搅拌中破碎？

在常规处理过程中，以及在具有标准叶轮的容器中以支持细胞贴壁和生长的速率搅拌，未发现对可溶性微载体的损坏。

17. 可溶性微载体怎样进行细胞传代？

建议通过离心或过滤方式去除微载体降解后细胞中的收获溶液。

为了优化微载体降解后的直接传代方案，我们建议测试在收获溶液不同稀释度下细胞在新的可溶性微载体上的贴壁情况。

同时，我们已验证了可通过直接加入新的可溶性微载体到培养系统的方式进行放大培养（如细胞的微载体球转球）。优化以添加新鲜微载体来扩增细胞的方案时，应考虑以下问题：

- 优化添加新鲜微载体的时机。我们建议在细胞亚汇合（50%-70%）时添加新的微载体。
- 改变搅拌方式为慢速搅拌或静止，以促进细胞移动。
- 应知晓工作体积的增加以及额外的微载体可能会影响混匀、微载体球相互碰撞和细胞迁移。

18. 如果我在微载体方面的经验有限，有推荐的起始方案吗？

首先，您应该在静止而不是搅拌的培养环境下验证细胞在可溶性微载体的贴壁。当微载体在水中溶胀并将水换成培养基后，我们建议在超低吸附表面的 6 孔板（康宁货号 3471）中每孔加入 2 mL 含 10 cm^2 微载体的培养基。注意不要让移液管接触到超低吸附表面，这可能会损坏表面包被，使细胞贴壁到孔板上。

然后，根据标准方案制备您的细胞悬液，每孔以 10,000 个细胞 /cm^2 微载体接种 2 mL 细胞悬液（共 100,000 个细胞），终体积为 4 mL。轻轻来回晃动培养板使细胞与微载体混合均匀，将其放置在 37℃二氧化碳培养箱或适合您细胞的培养环境中培养。每隔 30 分钟用显微镜观察细胞贴壁情况。评估不同的细胞接种密度、培养基添加剂（如有 / 无血清）和微载体表面，以确定支持细胞贴壁和铺展的最佳条件。

当细胞在可溶性微载体上达到约 70% 汇合度，验证微载体的降解和细胞释放。首先去除培养基，用 Dulbecco's 磷酸盐缓冲液（DPBS，康宁货号 21-031）洗涤一次，然后加入 1 mL 收获溶液（蛋白酶 +100 U/mL 果胶酶 +10 mM EDTA）。用显微镜监测 10-15 分钟内微载体的降解和细胞的释放。旋转培养板几次以促进单细胞悬液的形成。吹打混匀细胞悬液，取样进行细胞计数，并计算最终的细胞产量。在静止培养条件下，细胞在微载体上的倍增时间和扩增倍数预计与平面培养一致。

接下来，我们建议验证在转瓶搅拌培养下细胞的贴壁和生长。 以下是推荐的测试条件：

测试项目	推荐条件
转瓶	125 mL 一次性转瓶（康宁货号 3152）
培养的工作体积	50 mL
细胞接种密度	5,000 至 10,000 个细胞 /cm^2
可溶性微载体浓度	5 cm^2/mL（或 1 g/L）
搅拌速度	30-40 rpm

根据培养板静止培养实验的结果，确定细胞开始贴壁所需要的时间，将该时间应用到转瓶间歇搅拌方案：

- 如果细胞在静止条件下需要 30 分钟贴壁，那么建议在转瓶中接种后 12-24 小时内，每隔 30-60 分钟进行一次开 / 关搅拌循环。例如，将培养物以 30 rpm 混合 5 分钟，然后在 0 rpm 静置 30 分钟，总共重复该循环 12 至 24 小时，或直到 80%至 90%的细胞已经贴壁（通过监测培养基中未贴壁细胞的减少来确定）。建议间歇搅拌方案和连续搅拌方案都进行优化。

转瓶中可溶性微载体的降解及细胞收获可采用与静止培养相同的过程，并在收获期间以 30 rpm 搅拌培养物。

当放大培养到更大的转瓶时，我们推荐采用与 125 mL 一次性转瓶相同叶尖速度的搅拌速率。采用以下公式及叶轮直径计算叶尖速度：

叶尖速度（m/s）= DN/60，其中 D = 叶轮直径（m），N = rpm。

转瓶材料和尺寸	康宁货号	叶轮直径（m）
125 mL 一次性转瓶	3152	0.040
500 mL 一次性转瓶	3153	0.050
125 mL 玻璃转瓶	4500-125	0.040
250 mL 玻璃转瓶	4500-250	0.045
500 mL 玻璃转瓶	4500-500	0.059
1 L 玻璃转瓶	4500-1L	0.079

19. 如果细胞不贴壁到可溶性微载体上，怎么办？

首先，请参阅康宁关于可溶性微载体溶胀的实验方案（CLS-AN-467），以确保微载体在水中正确溶胀，并在溶胀后 1 周内使用。

其次，验证细胞在静止培养中能够贴壁到可溶性微载体。我们建议在超低吸附表面的 6 孔板（康宁货号 3471）中每孔加入 2 mL 含 10 cm^2 微载体的培养基。每孔以 10,000 个细胞 /cm^2 微载体接种 2 mL 细胞悬液（100,000 个细胞 / 孔），终体积为 4 mL。轻轻来回晃动培养板使细胞和微载体混合均匀，将其放置在加湿的 37℃二氧化碳培养箱或适合您细胞的培养环境中培养。每 30 分钟在显微镜下观察细胞贴壁情况，记录细胞贴壁所需的时间。

如果细胞仍不贴壁：

- 在贴壁阶段减少或去除培养基中的血清。
- 增加细胞接种密度。

优化静止培养中的细胞贴壁后，可在转瓶中重复这些条件。对于剪切敏感型细胞，可能需要对细胞贴壁期间的搅动速度/速率进一步优化。

20. 如果细胞在静止培养时可贴壁到可溶性微载体上，但搅拌培养（转瓶或生物反应器中）时，不像预期的那样扩大，怎么办？

微载体培养的优化可能需要评估培养基配方、搅拌速率、细胞接种密度及其它参数。强烈建议在进行放大之前优化搅拌培养条件。

21. 如果微载体不能完全降解，怎么办？

首先，请参阅康宁关于可溶性微载体溶胀的实验方案（CLS-AN-467），以确保微载体在水中正确溶胀并在溶胀后 1 周内使用。

其次，确认使用预热（> 25℃）的收获溶液溶解微载体。

然后，请参阅康宁关于可溶性微载体降解的实验方案（CLS-AN-466），证明裸微载体（无细胞）的可溶解性，确认收获溶液为含终浓度 100 U/mL 果胶酶（Sigma 货号 P2611; 3800 U/mL）和 10 mM EDTA（康宁货号 46-034-CI；0.5 M pH 8）的蛋白酶溶液。

最后，如果含有细胞的微载体仍不溶解：

- 在加入收获溶液之前，用 DPBS（康宁货号 21-031）洗涤 2 次，以减少或去除培养基残留组分（如血清、盐）对收获溶液中蛋白酶、果胶酶及 EDTA 功能的影响。
- 如果微载体上的细胞过度融合（如特别紧密的细胞单层或堆积的多层细胞），首先仅加入蛋白酶溶液（每克微载体 250 mL）以松动细胞层。当细胞开始变圆并暴露微载体表面，直接加入终浓度分别为 100 U/mL 和 10 mM 的果胶酶与 EDTA。

22. 如果在微载体溶解后观察到大的细胞团，该怎么办？

增加收获溶液中的蛋白酶浓度，延长收获时间，并确保收获溶液保持在蛋白酶的最佳温度。

23. 如果在微载体溶解后观察到大量的细胞串，该怎么办？

大的“串状”细胞团通常是细胞裂解的结果。将细胞收获期间的搅拌速度降低至使微载体悬浮所需的最小速度，缩短收获时间，或降低收获溶液中蛋白酶的浓度。

康宁可溶性微载体的溶胀

实验方案

图 1. 完全溶胀的可溶性微载体

康宁可溶性微载体以无菌干粉形式提供，其每克干重表面积为 5,000 cm^2。可溶性微载体在用于细胞培养之前必须用水进行溶胀。图 1 显示了完全溶胀后的微载体，其平均尺寸为 200-300 µm，密度为 1.01-1.03 g/cm^3。

使用以下方案溶胀康宁可溶性微载体：*

所需材料

微载体干粉	1 g	5 g	10 g
玻璃瓶	250 mL （货号 1395-250）	1 L （货号 1395-1L）	2 L （货号 1395-2L）
溶胀用水	150 mL	750 mL	1500 mL

第一步 准备一个硅化的无菌玻璃瓶 * 用于微载体溶胀。

第二步 在无菌条件下将可溶性微载体干粉倒入玻璃瓶中。

第三步 每克微载体干粉加入 150 mL 无菌水，轻轻搅拌溶液以确保溶胀均匀。请勿剧烈混合，避免空气进入。混合均匀后溶胀 10 分钟。在溶胀过程中不需要继续搅拌。

第四步 取样在显微镜下观察，与图 1 进行比较，确认是否完全溶胀。如有需要，溶胀后的可溶性微载体使用前可在 4℃储存最长 1 周。

第五步 静置微载体 30 分钟，将溶胀用水更换成培养基。吸弃水，加入所需体积的培养基即可。

* 更多信息，请参阅康宁可溶性微载体常见问题解答（CLS-BP-030）。

康宁可溶性微载体的降解

实验方案

可溶性微载体由钙离子交联的聚半乳糖醛酸（PGA）多聚物链构成。如图 1 所示，可溶性微载体的降解可通过添加 EDTA（螯合钙离子使聚合物交联不稳定）、果胶酶（降解 PGA 聚合物）和细胞消化蛋白酶（分解细胞和细胞外基质）进行。可溶性微载体可在 10-20 分钟内完全溶解。图 2 为显微镜下微载体的溶解和细胞释放，使用的收获溶液为 10 mM EDTA、100 U/mL 果胶酶和 TrypLE™ （Thermo Fisher，货号 A1217702）。

根据表 1 中的指导方案准备收获溶液 *。

第一步 将果胶酶（Sigma，货号 P2611；3800 U/mL）和 EDTA（康宁，货号 46-034-CI；0.5 M pH 8）直接加入到蛋白酶溶液中，确保果胶酶和 EDTA 最终浓度分别为 100 U/mL 和 10 mM。根据细胞类型选择蛋白酶溶液，并采用适合消化该细胞的浓度。

第二步 使用前，收获溶液需过滤除菌，并预热（> 25 ℃，或所用蛋白酶的最适温度）。

第三步 让微载体沉降在转瓶或生物反应器中，去除培养基。

第四步 用室温 1X DPBS（康宁，货号 21-031-CV）洗涤微载体，让微载体沉降并去除 DPBS 洗涤液。

第五步 根据表 1 中的指导方案，加入适量无菌、预热的收获溶液。

第六步 在室温或所用蛋白酶的最适温度下，采用使微载体悬浮所需的最小搅拌速度，轻轻搅拌 10-20 分钟。

第七步 取出细胞 - 微载体悬液样品，在显微镜下观察以确认微载体溶解和细胞释放情况，如图 2 所示。

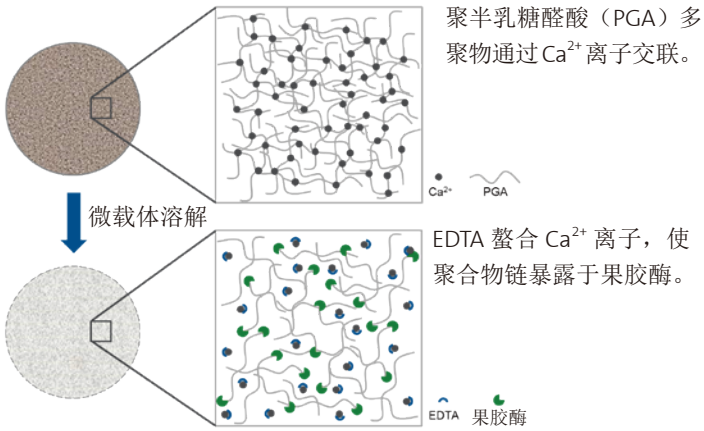


图 1. 微载体溶解原理

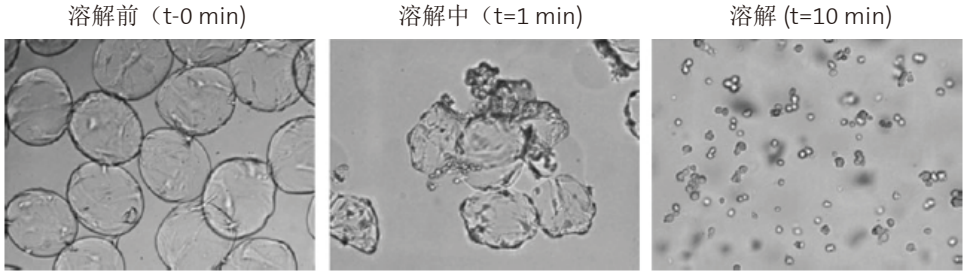


图 2. 可溶性微载体降解和人间充质干细胞的回收

表 1

可溶性微载体	1 g	5 g	10 g
收获溶液体积	250 mL	1250 mL	2500 mL
蛋白酶	238.4 mL	1192.1 mL	2384.2 mL
果胶酶	6.6 mL	32.9 mL	65.8 mL
EDTA	5.0 mL	25 mL	50 mL

* 更多信息，请参阅康宁可溶性微载体常见问题解答（CLS-BP-030）。

Human Mesenchymal Stem Cell Growth on Corning® Denatured Collagen Dissolvable Microcarriers in a 5L Bioreactor

Application Note

Jennifer Weber, Lori Romeo, and Paula Dolley-Sonneville
Corning Incorporated, Life Sciences
Corning, NY USA

Introduction

Microcarriers are 100 to 300 micron-sized beads that support the attachment and growth of adherent cells in stirred culture. They enable more cost-effective cell production by reducing the fixed costs (e.g., footprint and labor) and variable costs (e.g., media consumption) in large-scale manufacturing.

One challenge associated with microcarrier-based expansion is that microcarriers eventually must be removed or separated from the final product (e.g., cell, virus, antibody, etc.)[1,2]. Typically this is accomplished through filtration and/or centrifugation methods. Because of the broad size distribution of traditional microcarriers and the potential for bead breakage with use, several downstream microcarrier separation processes are required to remove residual microcarriers. These processes may create additional challenges resulting in filter fouling and loss of product.

To address these concerns, Corning developed a dissolvable microcarrier made from a polygalacturonic acid (PGA) polymer that is cross-linked via calcium ions. As shown in Figure 1A, microcarrier dissolution is achieved through the addition of a harvest solution containing EDTA, pectinase, and a standard cell culture protease. When calcium ions are chelated by EDTA, the PGA polymer destabilizes. Subsequent PGA polymer degradation is achieved by pectinase. The additional cell culture protease (e.g., trypsin, TrypLE™, Accutase®) breaks down cell-cell interactions and extracellular matrices, resulting in a single cell suspension in a solution of small sugar oligomers (Figure 1B).

This application note describes the use of Corning dissolvable microcarriers for the expansion and recovery of human mesenchymal stem cells (hMSCs) in 5L bioreactors. We demonstrate 7-fold expansion of hMSCs on 2 g/L denatured collagen dissolvable microcarriers in serum-containing medium. Continuous agitation was used to achieve uniform cell attachment and expansion, and microcarrier aggregate size was maintained through continuous gas sparging. Cells were recovered from

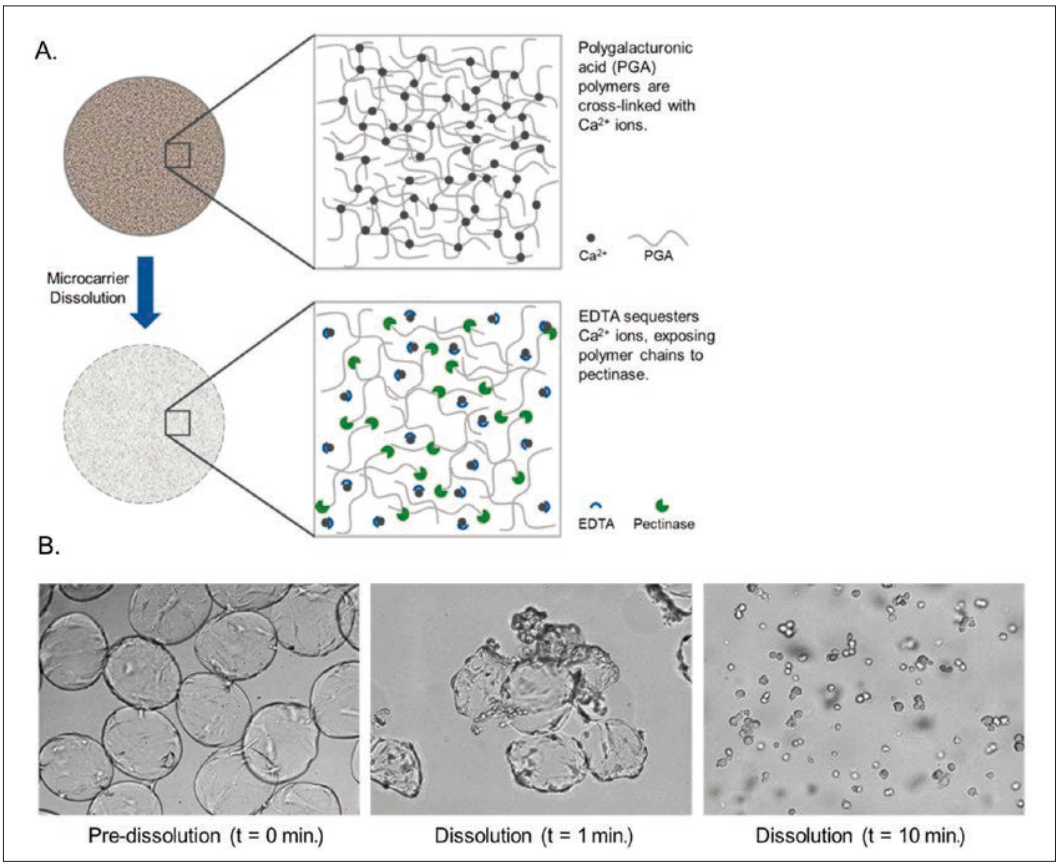


Figure 1. Description of Corning Dissolvable Microcarriers. Dissolvable microcarriers are made of polygalacturonic acid (PGA) polymer chains cross-linked via calcium ions. Dissolvable microcarriers have a surface area of 5,000 cm^2/gram; density of 1.02 to 1.03 g/cm^3, and diameter of ~250 micron. (A) Microcarrier dissolution is achieved with the addition of EDTA, pectinase, and a standard cell culture protease. (B) Microcarriers are completely dissolved within 10 to 15 minutes. Microscopy images show bead dissolution and hMSC release using a harvest solution of EDTA, pectinase, and TrypLE.

bioreactors using a harvest solution of EDTA, pectinase, and TrypLE™ with stirring for 20 minutes. The entire harvest process was completed by one operator in 1.5 hours and resulted in >95% cell recovery. Cells harvested from dissolvable microcarriers retained standard elongated morphology, normal karyotype, and multipotency, as measured by FACS analysis of phenotype and directed differentiation into osteocytes, chondrocytes, and adipocytes.

Methods

Dissolvable Microcarrier Preparation

The 10 g denatured collagen dissolvable microcarriers (Corning Cat. No. 4981) were aseptically hydrated in 1.5L of sterile water (Corning Cat. No. 25-055) in sterile, 2L glass bottles (Corning Cat. No. 1395-2L) that have been siliconized, for each bioreactor experiment according to the manufacturer's protocol. Water was replaced with culture medium by allowing microcarriers to settle and aspirating residual water without disturbing the microcarrier bead pack. Microcarriers were diluted in culture medium containing 1:1,000 antifoam (Thermo Fisher Cat. No. A10369-02) to a concentration of 2 g/L (surface area: 5,000 cm^2/gram) and aseptically transferred via a glass funnel into pre-sterilized, single-walled 5L glass bioreactors (Sartorius Biostat B® Twin bioreactors) fitted with one downward pumping 70 mm pitched blade impeller (Sartorius Cat. No. BB-8847401) and ring sparger with 1 mm downward bores (Sartorius Cat. No. UNIVESSEL-00013). Bioreactor cultures were allowed to equilibrate to 37°C at 90 rpm for 12 to 18 hours prior to cell addition. The pH (~7.4) and dissolved oxygen (DO, ~80%) levels were confirmed through offline measurements of samples (Nova Biomedical BioProfile® 400 Analyzer) before cell seeding.

Cell Preparation

Human mesenchymal stem cells (RoosterBio Cat. No. MSC-001) were thawed in Corning® CellBIND® surface-treated HYPER*Flask*® cell culture vessels (Corning Cat. No. 10034) and expanded in serum-containing medium (RoosterBio Cat. No. KT-001) for one passage to create a working cell bank according to manufacturers' protocols. For each bioreactor experiment, cells from the working cell bank were thawed into Corning CellBIND surface-treated HYPER*Flask* vessels and were harvested on day 4 using TrypLE (Thermo Fisher Cat. No. 12563029) for 12 minutes. Recovered cells were pelleted at 260 x g for 10 minutes, resuspended in fresh culture medium at 400,000 cells per mL (200 billion in 500 mL), and seeded directly (4,000 cells per cm^2) into equilibrated bioreactors containing dissolvable microcarriers. The final bioreactor working volume was 5L.

Cell Attachment to Dissolvable Microcarriers (0 to 24 hours)

Continuous agitation was used for cell attachment to dissolvable microcarriers. We observed cell attachment, but not complete spreading, after 10 cycles of 5 minutes at 100 rpm, followed by 20 minutes at 20 rpm. After ~5 hours, agitation was maintained at 100 rpm until 24 hours.

Cell Expansion on Dissolvable Microcarriers (24 to 96 hours)

To control microcarrier aggregate size with continued cell expansion, the agitation rate was increased to 120 rpm, and the DO was maintained at 50% using equal volumes of air and nitrogen sparge (25 to 40 ccm), with minimal supplementation of oxygen (<30 ccm) when needed. The pH was maintained at 7.4 through CO_2 sparge. The rate of gas addition at any given time was maintained at <0.02 vessel volumes per minute (vvm).

Sampling

The 10 mL samples were removed each day from the 5L bioreactor through an 1/8" ID dip tube with a syringe and transferred to an Ultra-Low Attachment (ULA) 6-well plate (Corning Cat. No. 3471) for microscopy (Zeiss Axiovert 40C) and spent media analysis (Nova Biomedical BioProfile 400 Analyzer). Cells were removed from dissolvable microcarriers for enumeration with a ViCell™ automated cell counter. Spent media was removed and samples were washed with 4 mL DPBS (Corning Cat. No. 21-031) and 2 mL of pre-warmed (>25°C) harvest solution was added. To prepare the harvest solution, a 5X solution of TrypLE was made from equal volumes of a 10X stock solution (Thermo Fisher Cat. No. A1217702) and DPBS. Pectinase (Sigma Cat. No. P2611) and EDTA (Corning Cat. No. 46-034-CI) were directly supplemented to the 5X TrypLE solution to a final concentration of 100 U/mL and 10 mM, respectively. Plates containing microcarrier cultures in harvest solution were placed in a 37°C incubator for 10 minutes. Cultures were gently mixed using a 5 mL Stripette™ serological pipet (Corning Cat. No. 4487) and observed under the microscope to confirm complete microcarrier dissolution and a single cell suspension. The sample was again mixed and 700 µL was transferred to a ViCell cup for enumeration.

Microcarrier Dissolution and Cell Recovery

Cells were harvested from day 4 bioreactor cultures by turning off all bioreactor controls and allowing microcarriers to settle for 15 minutes; this resulted in a settled bead pack volume of ~1L. Spent culture medium was removed through 1/8" ID dip tube, and 2 washes with 1L DPBS were performed to remove residual serum. A pre-warmed, filter sterilized (500 mL vacuum filtration system, Corning Cat. No. 430769) harvest solution was prepared as a 3X concentrate as follows: pectinase and EDTA were added to 500 mL of 10X TrypLE to a final concentration of 200 U/mL and 20 mM, respectively. The 500 mL of 3X harvest solution was added to the ~1L packed bead bed, resulting in a final volume near 1.5L allowing for a completely submersed impeller. Microcarrier dissolution was facilitated through gentle mixing at 80 rpm for 10 minutes; then agitation was increased to 100 rpm for an additional 10 minutes, resulting in a near single cell suspension. Cell suspensions were removed from bioreactors through a dip tube and total cell yields were calculated using a ViCell automated cell counter.

Cell Characterization

Aliquots of recovered cells were pelleted at 260 x g, washed with DPBS, and resuspended in CryoStor® CS5 freeze media (BioLife Solutions Cat. No. 205102) for later analysis.

For cell differentiation into chondrocytes, osteocytes, and adipocytes, cells were thawed, washed in culture medium to remove freezing medium, and re-seeded in Corning CellBIND surface 6-well plates (Corning Cat. No. 3335) in StemPro® differentiation media according to manufacturer's protocols (Thermo Fisher

Cat. Nos. A10071-01, A10072-01, and A10070-01). After 2 to 3 weeks, differentiated cells were fixed in 2% paraformaldehyde and stained for markers of differentiation: chondrocytes (chondrogenic pellets via Alcian Blue, Sigma Cat. No. TMS-010-C), osteocytes (calcium deposits via Alizarin Red, Sigma Cat. No. TMS-008-C), and adiptocytes (lipid droplets via Oil Red O, Sigma Cat. No. O1516-250ML).

For quantitative analysis of cell phenotype, recovered cells were thawed, immunostained for positive (CD73, CD90, and CD105 [BD Biosciences Cat. Nos. 550256, 555593, 555690, respectively]), and negative (CD14 [MilliporeSigma Cat. No. MAB1219], CD34 [BD Biosciences Cat. No. 555820]) surface markers, and the percent of viable cells expressing each marker were assessed via flow cytometry. We observed more consistent expression of CD105 when thawed cells were replated in flasks for a few hours until fully spread; replated cells were harvested from flasks using TrypLE™ and directly immunostained according to standard protocols. Briefly, cells were diluted to 1 x 10^6 cells/mL in 1X DPBS supplemented with 10% heat-inactivated serum (Corning Cat. No. 35-011-CV) (blocking buffer) and 1:1,000 propidium iodide (Millipore Sigma Cat. No. P4864) and incubated in the dark for 20 minutes at room temperature. Cells were washed twice with 1X DPBS, resuspended in blocking buffer at 0.5 x 10^6 cells/50 µL, and incubated with primary antibodies (10 µg/mL) or corresponding IgG1 isotype control (BD Biosciences Cat. No. 554121) for 30 minutes at 4°C in the dark. Cells were washed in staining buffer containing BSA (BD Biosciences Cat. No. 554657) and then incubated with secondary antibody (1:1,000 of Alexa Fluor® 488 goat anti-mouse IgG1 [Thermo Fisher Cat. No. A11001]) for 30 minutes at 4°C in the dark. Stained cells were washed once and resuspended in staining buffer for processing on a BD FACSCalibur™ flow cytometer. During acquisition, 30,000 events/sample were collected and analyzed using the BD CellQuest™ Pro software.

For analysis of cell karyotype, cells were submitted as live cultures in flasks for G-banded karyotype analysis at WiCell Cytogenetics Laboratory.

Results

Cell Attachment to Dissolvable Microcarriers

To demonstrate attachment of human mesenchymal stem cells (hMSCs) on microcarriers in bioreactors, we first determined the best agitation conditions that would support a uniform cell attachment across all microcarriers. Based on preliminary results with dissolvable microcarriers in spinner flasks, several agitation protocols in the bioreactor were investigated for the cell attachment phase: no agitation, intermittent cycles of settling and mixing, and completely continuous agitation. A minimum agitation speed that supported complete suspension of all microcarriers was determined experimentally by observing the microcarrier suspension throughout the working volume at different agitation speeds. Based on the following bioreactor configuration: one 70 mm pitched blade impeller positioned as low as possible on the impeller shaft, 5L working volume, and 2 g/L microcarrier concentration (10 cm^2/mL), a minimum agitation speed of 100 rpm supported complete suspension of the microcarriers throughout the working volume.

When cells were added to bioreactors containing suspended microcarriers, the culture was mixed at 100 rpm for 5 minutes before initiating one of the three agitation protocols described above: no agitation (0 rpm), continuous agitation (100 rpm), or intermittent cycles of 20 rpm for 20 minutes then 100 rpm for 5 minutes. It was expected that no agitation during cell attachment would promote an attachment rate similar to what is observed in planar cultureware; however, because the microcarriers would settle in the absence of agitation, cells would primarily attach to only those microcarriers exposed at the top of the bead pack, resulting in a non-uniform distribution of cells across the microcarrier population. In contrast, it was expected that a completely continuous agitation at 100 rpm would support the most uniform cell attachment to all suspended microcarriers, but the cell attachment period may be prolonged or potentially inhibited due to the hydrodynamic shear. For these reasons, intermittent cycles of low- and high-speed stirring were investigated to promote phases of bead settling and mixing. Based on the observation in planar flasks that cell attachment occurs within 20 minutes and complete spreading is achieved within a few hours, a 20-minute period of low agitation (20 rpm) was implemented to encourage cell attachment.

To achieve a more uniform attachment across all microcarriers, the culture was mixed at the minimum speed (100 rpm) required to suspend all the microcarriers for the minimum time (5 minutes) required to achieve a complete microcarrier suspension. This intermittent mixing protocol was continued until cell spreading was observed. To evaluate the effect of each agitation protocol on cell attachment, 10 mL samples were removed every hour to monitor cell attachment and spreading via microscopy. We recommend investigating different intermittent mixing cycles depending on the cell type, media condition (+ or - serum), microcarrier surface (charged vs. peptide/protein), cell seeding density, or microcarrier concentration[1,3,4].

As expected, no agitation (0 rpm) and constant agitation (100 rpm) did not support the desired uniform cell attachment with spreading. The culture exposed to no agitation contained both sparse, large cell-microcarrier aggregates with fully spread cells and numerous single, bare (no cells) microcarriers. This could be a result of cell-cell clustering and subsequent settling to the top of the microcarrier bead pack before attachment and spreading. The culture exposed to continuous agitation, in contrast, had better cell attachment uniformity, but very few cells had attached to the microcarriers even after 24 hours. The attachment protocol involving intermittent mixing supported the most uniform attachment of the majority of cells. Based on hourly microscopy images from samples, cyclic periods of mixing were continued for a total of 5 hours; at this point, very few non-attached cells were observed, and spreading of attached cells had begun (Figure 2). After the 5-hour attachment period, the culture was exposed to constant agitation at the minimum agitation speed (100 rpm) required to suspend the microcarriers. By 24 hours post-seeding, fully elongated cells (Figure 3) were observed and nearly 100% of cells were attached, based on cell enumeration from representative 10 mL samples.

Cell Expansion on Dissolvable Microcarriers

Based on preliminary experiments in small-scale spinner flasks, hMSC expansion was expected to be most impacted by cell-microcarrier aggregate size and aggregate settling as a result of increasing size. Several methods were investigated to reduce microcarrier aggregate size by promoting better mixing such as: increasing agitation speed, utilizing different impeller shapes or designs, adding a second impeller, and controlling gas sparge rate[2]. Limited success was observed when increasing the agitation speed of a single impeller and with adding a second impeller; in both situations, microcarrier aggregate size and distribution were able to be maintained early in the culture period but could not be sustained at higher cell confluence without a negative effect on cell viability, most likely resulting from an increase in hydrodynamic shear.

Next, the effect of gas sparging rate on microcarrier cluster formation was investigated, based on methods implemented in large-scale vaccine processes with suspension cells5. Two scenarios for gas sparging were evaluated to maintain a dissolved oxygen (DO) level of 50%: (1) intermittent sparging of air or oxygen gas when the DO level reached the setpoint; and (2) constant gas sparging starting 24 hours post-seeding using a mixture of air and nitrogen with oxygen supplementation when needed. As shown in Figure 4, a constant flow of gas delayed the formation of cell-microcarrier aggregates and promoted a more uniform microcarrier aggregate size and distribution throughout the working volume of the culture. Specifically, the air/nitrogen flow rate was adjusted relative to the oxygen flow rate to ensure that the combined gas flow from 24 to 96 hours post-seeding was 0.01 to 0.02 vessel volumes per minute (vvm). Using this approach, hMSC growth on denatured collagen dissolvable microcarriers was evaluated in 3 independent 5L bioreactor runs. As shown in Figure 5, a cell density of 35,000 cells/cm^2 (350,000 cells/mL) was achieved by day 4. Nutrient depletion and metabolite accumulation in the culture medium were monitored daily; these results indicated that medium replenishment was not required during the 4-day culture period. Representative images of cell confluence and microcarrier aggregate size are shown.

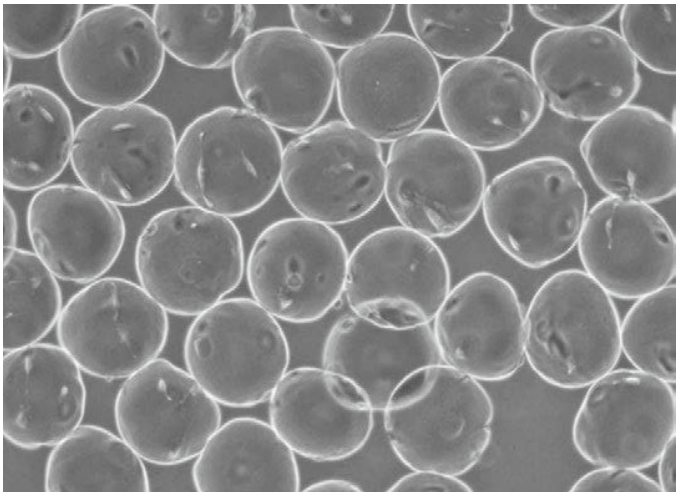


Figure 2. hMSC attachment to Corning® dissolvable microcarriers after 5 hours. Five hours after cell seeding, a 10 mL sample from the bioreactor was removed and transferred to an Ultra-Low Attachment (ULA) 6-well plate. Cell attachment and uniformity of distribution were assessed via microscopy. A representative image at 5X magnification is shown.

Even though continuous gas sparging was implemented to better control microcarrier aggregate size, a gradient of microcarrier clusters was still observed throughout the 5L liquid volume, which changed based upon the daily rate of cell growth. Therefore, acquiring a representative sample from the bioreactor at a defined dip tube location during the 4-day culture period was challenging. To address this concern and obtain a more accurate assessment of total yield on harvest day, samples were removed at different dip tube locations within the bioreactor. 10 mL samples were removed from 7 dip tube locations (Figure 6A). Cells were harvested from each sample and averaged to obtain a cell concentration (~27,000 cells/cm^2 or 7-fold expansion) more representative of the full culture volume (Figure 6B). This value was then compared to the number of cells recovered from the full 5L volume after microcarrier dissolution.

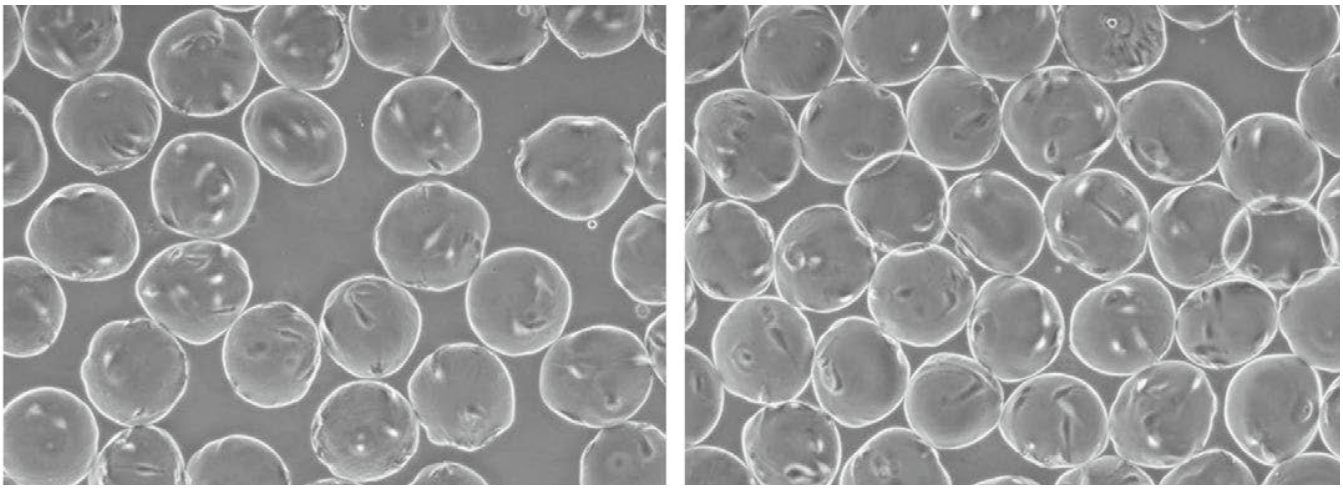


Figure 3. hMSC attachment to Corning dissolvable microcarriers after 24 hours. Twenty-four hours after cell seeding, a 10 mL sample from the bioreactor was removed and transferred to an Ultra-Low Attachment (ULA) 6-well plate. Cell attachment and uniformity of distribution were assessed via microscopy. Representative images at 5X magnification are shown.

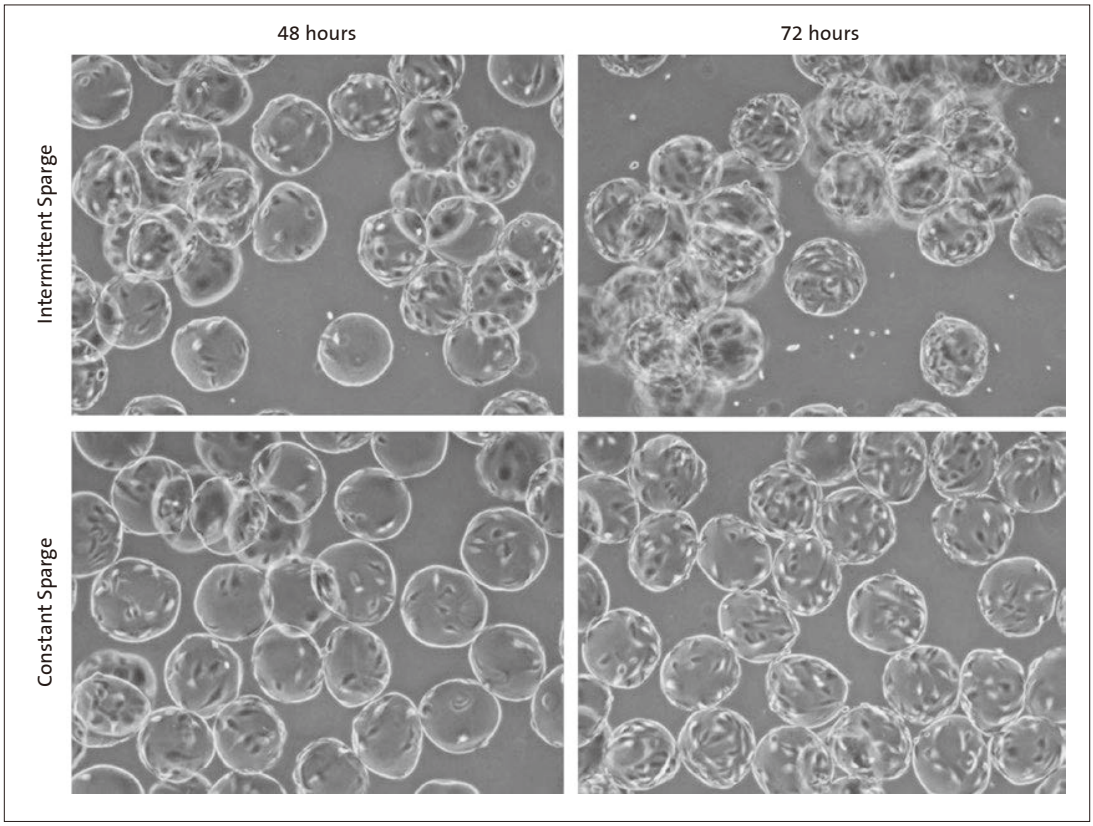


Figure 4. A comparison of gas flow rate on microcarrier aggregate size. Two gas flow rate protocols, intermittent sparge and constant sparge, were investigated to control microcarrier aggregate size with continued cell expansion. As shown, intermittent injections of gas through a ring sparger to maintain a DO set point resulted in microcarrier aggregates (~10 beads) by 48 hours, and the aggregate size continued to increase with cell growth by 72 hours (top images). In contrast, continuous gas sparging decreased the amount and size of microcarrier aggregates (bottom images), resulting in better cell surface area utilization of individual microcarriers. Representative images are shown at 5X magnification.

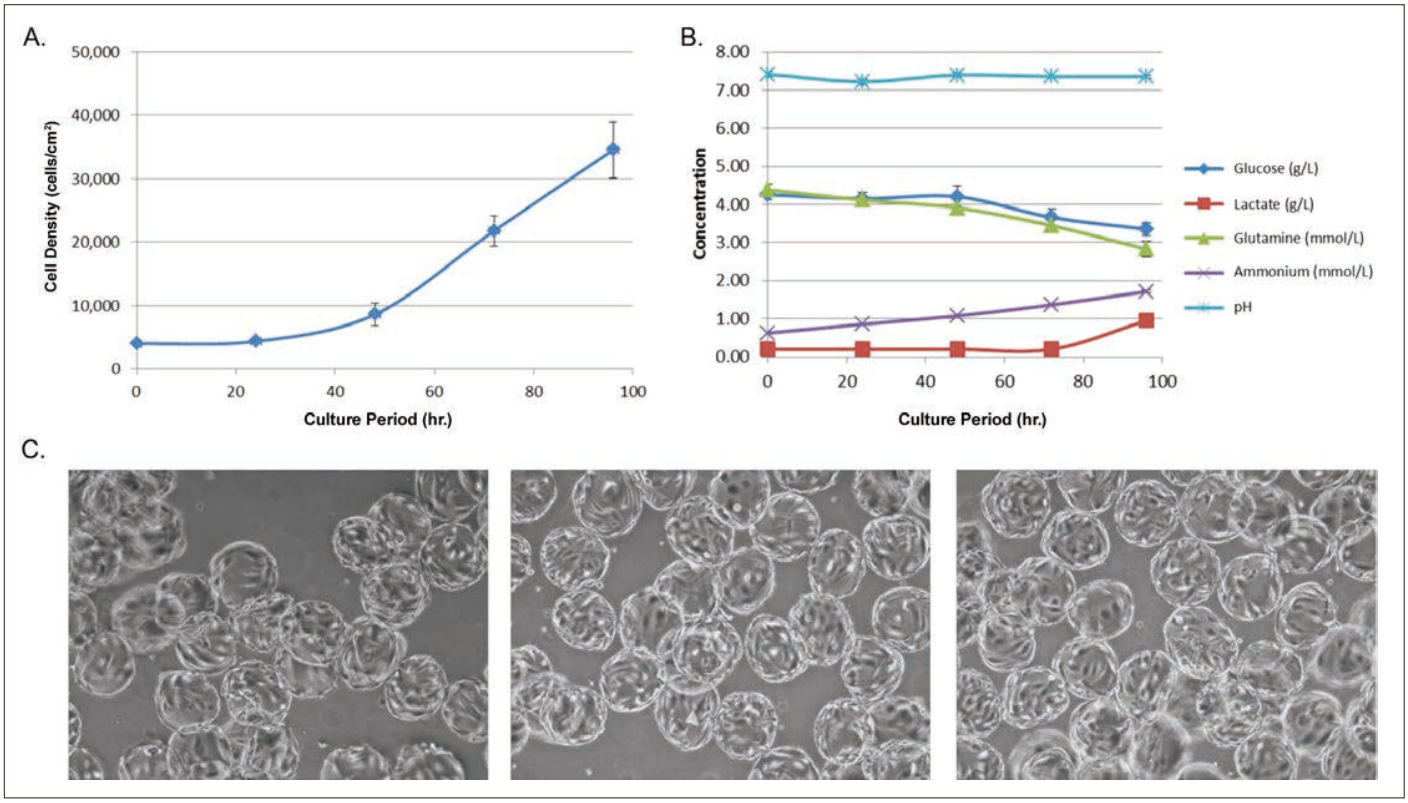


Figure 5. Cell expansion on dissolvable microcarriers in 5L bioreactors. (A) hMSCs were seeded on denatured collagen dissolvable microcarriers in 5L bioreactors, and daily samples were removed to assess cell growth; error bars represent the standard deviation of the average cell density per day from 3 independent bioreactor experiments. (B) Daily spent media samples were analyzed for pH, nutrient (glucose, glutamine) consumption, and metabolite (lactate, ammonium) accumulation. (C) Representative 5X images from the 3 bioreactor cultures show ~70% confluent hMSCs on dissolvable microcarriers at day 4.

Microcarrier Dissolution and Cell Recovery

Cells were harvested from day 4 bioreactor cultures by allowing microcarriers to settle, removing spent culture medium, and washing twice with DPBS to reduce the residual serum concentration. Next, a concentrated harvest solution containing pectinase, EDTA, and TrypLE™ were added, and stirring at 80 rpm to gently mix the culture. After 10 minutes, a sample was removed to check the dissolution process; no remaining microcarriers were seen, but cells remained in small clusters and slightly elongated. The agitation speed was increased to 100 rpm for an additional 10 minutes to promote a single cell suspension and fully rounded cells (Figure 7). The cell suspension was removed from the bioreactor, and the total cell yield and viability were calculated using a ViCell™ automated cell counter. The entire harvest process was completed by one operator in 1.5 hours. Comparison of the final cell yield from the full volume harvest to that based on the calculated average from the 7 dip tube locations, an average cell recovery of 96% (~1.4 billion cells/bioreactor) and cell viability of 93% was calculated for 3 independent bioreactor experiments.

Characterization of Recovered Cells

To confirm that hMSCs grown on dissolvable microcarriers retain their standard elongated morphology and ability to migrate in planar cultureware, a sample of cells on microcarriers (before microcarrier dissolution) was re-plated in 6-well plates, and cells were allowed to migrate from microcarriers to the plate surface (Figure 8). Similarly, a sample of cells recovered after microcarrier dissolution were directly replated in 6-well plates. As shown in Figure 8, cells successfully migrated from the dissolvable microcarriers and displayed elongated cell morphology similar to those cells seeded directly after microcarrier dissolution.

Secondly, to confirm the multipotency of cells recovered from dissolvable microcarriers, cells were immunostained for phenotypic markers (positive: CD73, CD90, CD105; negative: CD34, CD14) and also re-plated for directed differentiation into adipocytes, osteocytes, and chondrocytes (Figure 9). Cells recovered from dissolvable microcarriers in 3 independent bioreactor experiments (red bars) retained expression of phenotypic markers, as measured by flow cytometry, comparable to both the input cells from Corning® HYPER*Flasks*® used to seed the bioreactor cultures (blue bars) and to same-passage control cells harvested from T-flasks (green bars). Similarly, cells exposed to differentiation media for 2 to 3 weeks positively stained for markers of differentiation: lipid droplets via Oil Red O (adipocytes), calcium deposits via Alizarin Red (osteocytes), and chondrogenic pellets via Alcian Blue (chondrocytes) as shown. Lastly, recovered cells retained normal 46, XX karyotype (data not shown).

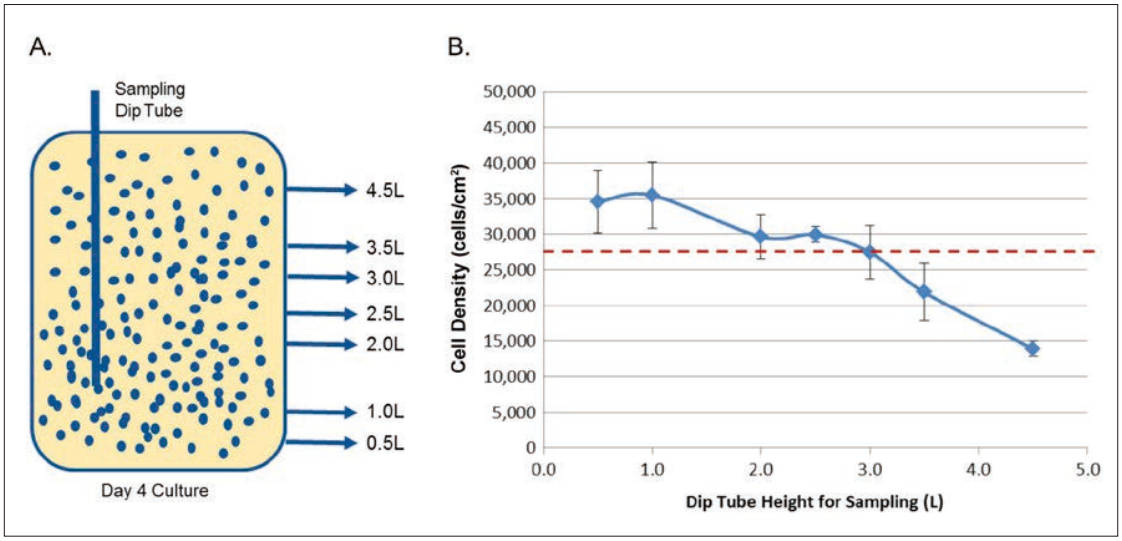


Figure 6. Investigation of dip tube position for bioreactor sampling. (A) Day 4 bioreactor cultures were sampled at various locations (or volume heights) within the bioreactor as shown by the position of the 7 blue arrows. (B) cells were enumerated from samples taken at these locations; error bars represent the standard deviation of 3 independent bioreactor runs. The average cell density across the 7 samples was calculated as 27,000 cells/cm^2 and shown by the red dotted line.

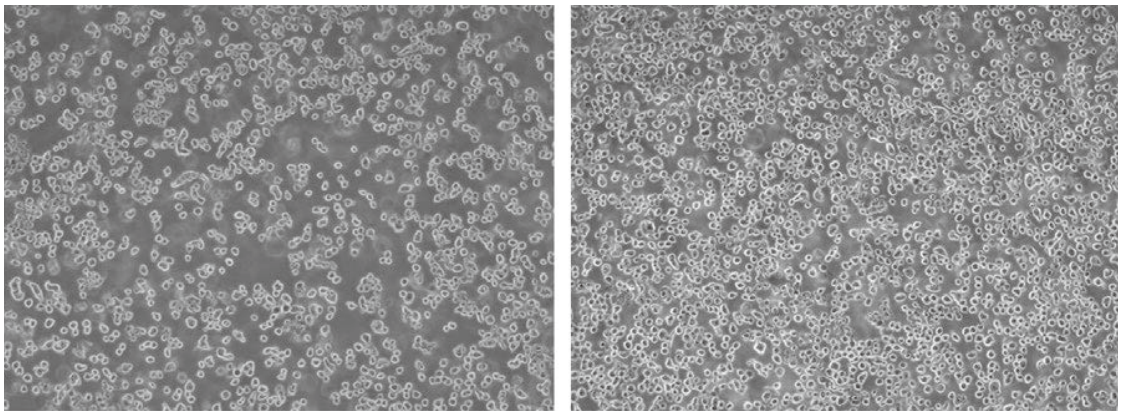


Figure 7. Cell suspensions after microcarrier dissolution. Microcarriers were dissolved in 5L bioreactors using a harvest solution of TrypLE, pectinase, and EDTA. Representative images of recovered hMSC suspensions post microcarrier dissolution are shown at 5X magnification.

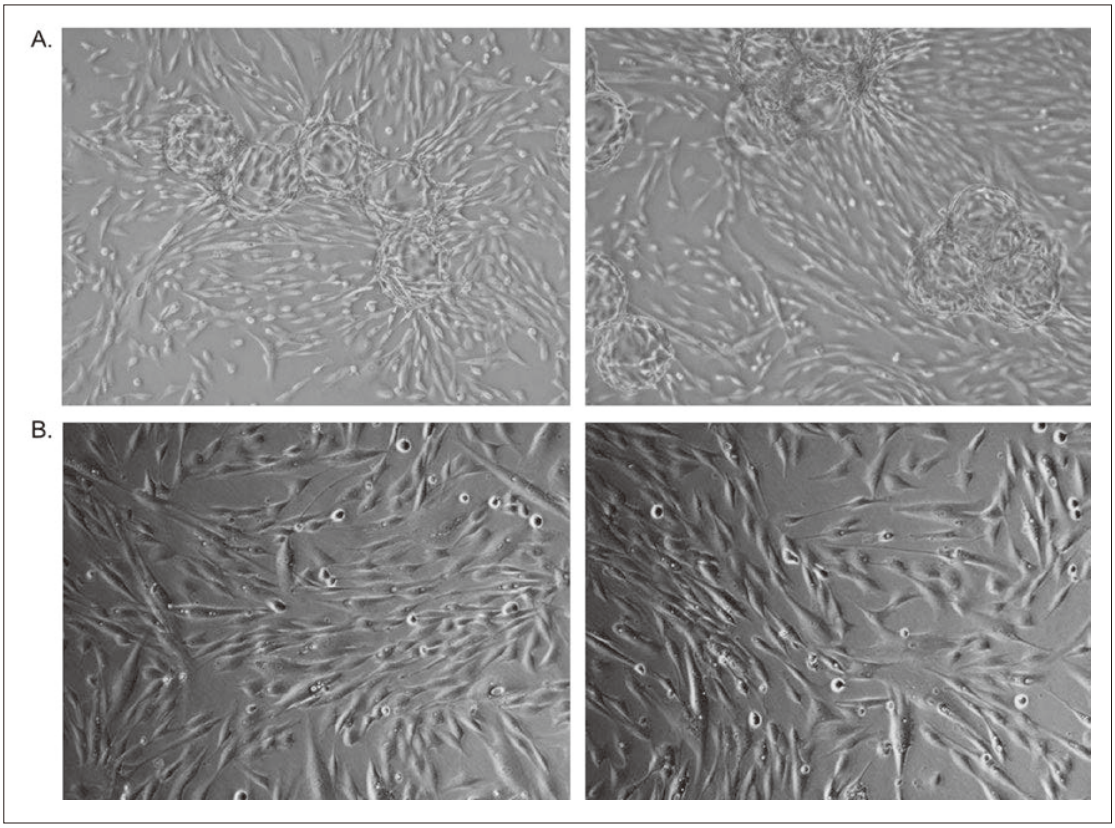


Figure 8. Standard hMSC morphology is maintained after culture on dissolvable microcarriers. (A) Before microcarrier dissolution, cells on microcarriers were transferred to 6-well plates, and cells migrated from microcarriers to the plate surface. (B) Similarly, cells recovered after microcarrier dissolution were directly re-plated in 6-well plates. Both cultures displayed typical hMSC elongated morphology. Representative images are shown at 5X (A) and 10X (B) magnifications.

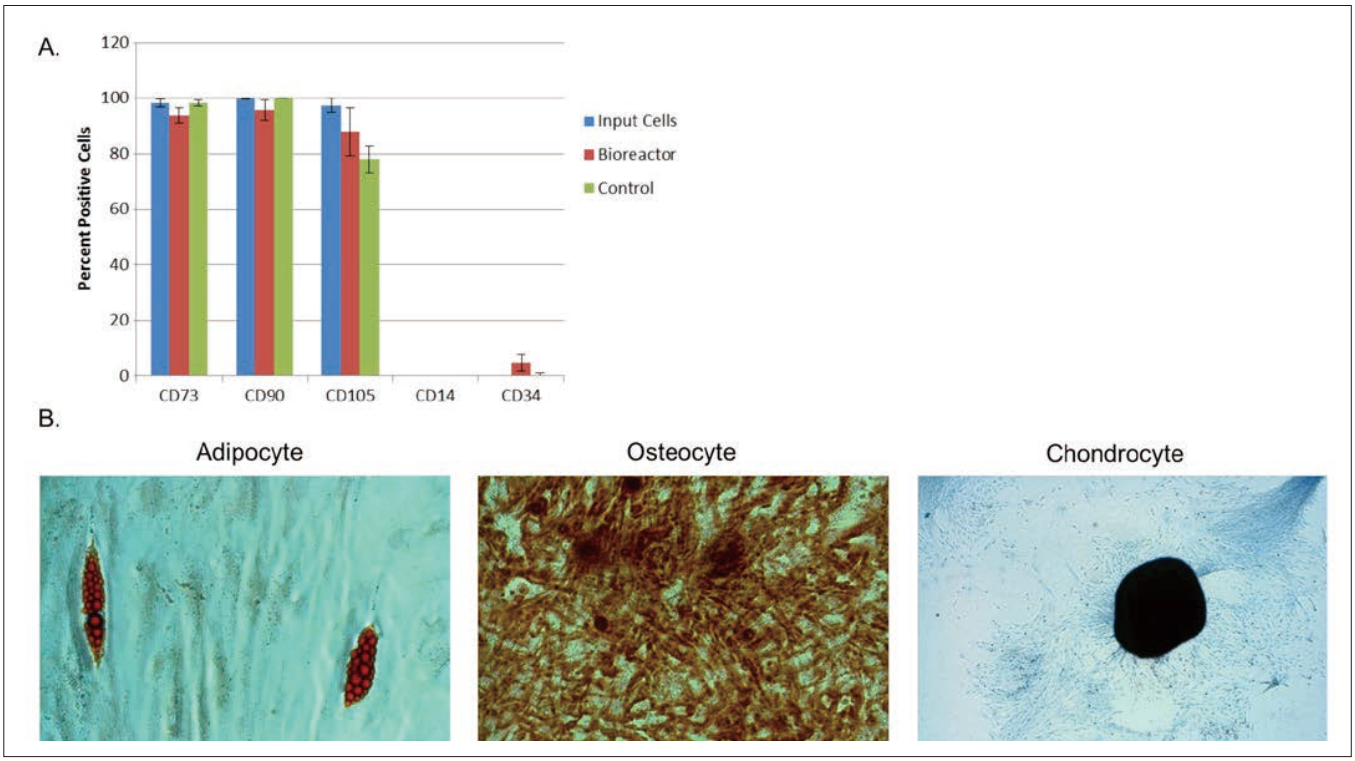


Figure 9. Characterization of cell multipotency after culture on dissolvable microcarriers. Cells recovered from dissolvable microcarriers in 3 independent bioreactor experiments were immunostained for phenotypic markers and analyzed by flow cytometry. (A) Cells recovered from microcarriers (red bars) retained high expression of positive markers of phenotype: CD73, CD90, and CD105 comparable to both the input cells from Corning® HYPER*Flasks*® used to seed the bioreactor cultures (blue bars) and to same-passage control cells harvested from T-flasks (green bars). Also, recovered cells were re-plated in 6-well plates for media-induced differentiation into adipocytes, osteocytes, and chondrocytes. (B) Cells exposed to differentiation media for 2 to 3 weeks positively stained for markers of differentiation: lipid droplets via Oil Red O (adipocytes, 20X magnification), calcium deposits via Alizarin Red (osteocytes, 10X magnification), and chondrogenic pellets via Alcian Blue (chondrocytes, 2.5X magnification).

Summary

We demonstrated 7-fold expansion of hMSCs on denatured collagen dissolvable microcarriers in 5L bioreactors. Continuous agitation was used to achieve uniform cell attachment and expansion, and microcarrier aggregate size was maintained through continuous gas sparging. We recovered 96% of cells from dissolved microcarrier cultures using a harvest solution of EDTA, pectinase, and TrypLE™, and the recovered cells retained standard elongated morphology, normal karyotype, and multipotency, as measured by FACS analysis of phenotype and directed differentiation into adipocytes, osteocytes, and chondrocytes.

References

1. Viswanathan S, Hematti P, eds. 2017. Mesenchymal stromal cells: Translational pathways to clinical adoption. Ch. 6,139-178.
2. Schnitzler AC, Verma A, Kehoe DE, Jing D, Murrell JR, Der KA, Aysola M, Rapiejko PJ, Punreddy S, Rook MS, 2016. Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: Current technologies and challenges. Biochemical Engineering Journal. 108:3-13.
3. de Soure AM, Fernandes-Platzgummer A, da Silva CL, Cabral JMS, 2016. Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells. Journal of Biotechnology. 236:88-109.
4. Strathearn KE, Pardo AMP, 2014. Parameters to consider when expanding cells on Corning microcarriers. Corning Application Note CLS-AN-243. https://www.corning.com/worldwide/en/products/life-sciences/products/bioprocess/microcarriers.html.
5. Hu W, Berdugo C, Chalmers JJ, 2011. The potential of hydrodynamic damage to animal cells of industrial relevance: Current understanding. Cytotechnology. 63:445-460.

Expansion of Human Mesenchymal Stem Cells on Corning® Synthemax™ II-coated Corning Dissolvable Microcarriers in a Serum-free Cell Culture Medium

Application Note

Jeffery J. Scibek and Paula Dolley-Sonneville
Corning Research and Development Corporation
Corning, NY USA

Introduction

Human mesenchymal stem cells (hMSCs) are currently the most common adult stem cell type used for cell therapy applications due to their regenerative properties and ability to differentiate into multiple cell lineages (adipocyte, chondrocyte, and osteocyte)[1,2]. Traditionally, hMSCs have been cultured on 2-dimensional cell culture platforms using serum-containing medium. Although these platforms can be used successfully for small-scale expansion of hMSCs, other platforms will be required to generate the quantity of cells required to support the increasing number of clinical trials utilizing hMSCs and the subsequent large-scale production[3]. Microcarriers represent a viable solution by enabling anchorage-dependent stem cells to be cultured in suspension, enabling significant scale-up in bioreactors.

A critical parameter that needs to be considered when selecting the optimal microcarrier is the intended use of the product. For cell therapy applications, a viable, functional cell is the desired product. Unfortunately, most commercially available microcarriers have significant disadvantages that hinder their use for cell therapy applications. Most microcarriers are composed of a solid matrix which necessitates the need for the microcarrier to be separated from the dissociated cells during downstream processing. This separation step adds complexity and expense to the overall production process. Another limitation observed with microcarriers is the difficulty to efficiently dissociate cells following cell expansion due to the strength of cell attachment to the microcarriers. For these microcarriers, overall cell yield is significantly reduced and, more importantly, the functionality of the harvested cells may be impacted by the harsh cell dissociation strategies that are required.

Corning dissolvable microcarriers provide an ideal solution for the large-scale expansion of hMSCs for bioprocess applications. Corning dissolvable microcarriers are composed of cross-linked polysaccharide polymers that can be dissolved during the cell harvest step. The ability to completely dissolve the microcarrier results in simpler downstream purification processes and eliminates the need to physically separate the cells and microcarriers. Corning dissolvable microcarriers are quickly dissolved following the addition of an animal component-free harvest solution. A cell-dissociation enzyme can be added during the harvest step in order to facilitate a single-cell suspension.

Here we describe a user-friendly protocol for the expansion of hMSCs in serum-free medium. hMSCs were cultured on Corning Synthemax™ II-coated dissolvable microcarriers in StemPro® MSC SFM (serum-free medium) demonstrating efficient cell attachment and expansion. Cells were cultured for up to 7 days resulting in cell yields exceeding 40,000 cells/cm^2 (>7-fold expansion). During the cell harvest step, the microcarriers were completely dissolved within 10 minutes resulting in a single-cell suspension. The harvested cells were characterized for marker expression, karyotype, and the ability to differentiate into adipocytes, chondrocytes, and osteocytes. These results demonstrate that Corning dissolvable microcarriers are an ideal scalable platform for the expansion and efficient harvest of functional hMSCs in serum-free medium.

Methods and Materials

Cell Preparation

Human bone marrow-derived mesenchymal stem cells were purchased from RoosterBio (RosterBio Cat. No. MSC-001). Cells were adapted to StemPro MSC SFM (Thermo Fisher Cat. No. A1033201) and expanded in Corning CellBIND® CellSTACK® 2-chamber vessels (Corning Cat. No. 3310) that were coated with Corning Synthemax II-SC at 5 mg/cm^2 (Corning Cat No. 3535). A cell bank was established by cryopreserving the cells in CryoStor® CS5 (STEMCELL Technologies Cat. No. 07933).

To generate cells for microcarrier cultures, hMSCs were thawed and seeded directly into CellBIND CellSTACK 2-chamber vessels (coated with Synthemax II) at 5,000 cells/cm^2 in 300 mL StemPro MSC SFM. The cells were incubated for 5 days in a cell culture incubator with a media exchange (300 mL) on day 3. To harvest, cells were washed once with 100 mL DPBS (Corning Cat. No. 21-031-CM) and then incubated with 50 mL TrypLE™ Select Enzyme (10X) (Thermo Fisher Cat. No. A12177-01) for 8 minutes. Following cell dissociation, cells were centrifuged at 200 xg for 5 minutes and then resuspended in StemPro medium.

Dissolvable Microcarrier Protocols

hMSCs were cultured on Synthemax II-coated Corning dissolvable microcarriers (Corning Cat No. 4983) in StemPro MSC SFM in a 1L Corning ProCulture glass spinner flask (Corning Cat. No. 4500-1L). The final culture volume in each flask was 600 mL. The total microcarrier surface area per flask was 3,000 cm^2 (5 cm^2/mL).

Cells were seeded at a concentration of 6,000 cells/cm^2. Prior to use, the glass spinner flasks were treated with Sigmacote® (MilliporeSigma Cat. No. SL2-25ML), washed with water, and autoclaved to minimize sticking of microcarriers.

Microcarrier Hydration

Corning® dissolvable microcarriers are supplied as a sterile, dry powder and must be hydrated prior to use. To hydrate microcarriers for a single 1L glass spinner flask (GSF), 3,000 cm^2 (0.6 g) dissolvable microcarriers were aseptically transferred to a sterile container. Next, 90 mL sterile water (Corning Cat. No. 25-055-CM) was added to the bottle (150 mL water per gram microcarriers), and the mixture was gently swirled in order to resuspend the microcarriers. For additional information, see Hydration of Dissolvable Microcarriers Protocol (CLS-AN-467). The microcarriers were incubated for 10 minutes to allow complete hydration and then the water was removed from the settled microcarrier bed. **Note:** the microcarriers can be washed in StemPro® MSC SFM in order to minimize dilution of the culture medium due to residual water from the hydration step. Next, the microcarriers were resuspended in StemPro MSC SFM to a final volume of 60 mL (50 cm^2/mL microcarrier concentration).

Preparation of Dissolvable Microcarrier Culture in 1L Glass Spinner Flasks

First, 420 mL of StemPro MSC SFM was added to the 1L GSF. Next, 60 mL of microcarrier suspension (at 50 cm^2/mL) was added to the GSF. The flask was then incubated in a cell culture incubator for ~30 minutes to allow the media to equilibrate. During the equilibration step, hMSCs were harvested from Corning CellBIND® CellSTACK® 2-chamber vessels as described above. Cells were seeded at a density of 6,000 cells/cm^2 ($1.8x10^7$ cells/GSF). The total volume for the cell addition step was 120 mL. If less than 120 mL of cell stock is added to the 1L GSF, the appropriate volume of StemPro MSC SFM should be added to the GSF to bring the total cell addition volume to 120 mL. This will result in a final culture volume of 600 mL for the 1L GSF.

Cell Attachment Phase

An intermittent mixing protocol was employed during the cell attachment phase. After the addition of cells to the 1L GSF, the culture was mixed for 2 minutes at 22 rpm. Next, the culture was incubated at 0 rpm (static phase) for 30 minutes. This process was repeated for 8-12 hours (16-24 cycles). Cell attachment efficiency should exceed 80% at the end of the attachment phase. Cell attachment was monitored by filtering a 1 mL sample of the microcarrier culture through a 35 µm cell strainer (Corning Cat. No. 352235) and quantifying the cell concentration of the flow through.

Cell Expansion Phase

Following the cell attachment phase, the microcarrier culture was mixed continuously at 16 rpm. During the cell expansion phase, the mixing speed was increased daily in an attempt to minimize microcarrier aggregation. The mixing speed was increased as follows: 16 rpm (following cell attachment), 16 rpm (day 1), 22 rpm (day 2), 27 rpm (day 3), 32 rpm (days 4-7). Half-volume media exchanges were performed on days 3 and 5. To exchange the media, the microcarriers were allowed to settle in the 1L GSF for 3-5 minutes. Next, 300 mL of the spent media was removed and 300 mL fresh StemPro MSC SFM was added. The 1L GSF was then returned to the stir platform in the cell culture incubator.

Microcarrier Dissolution and Cell Release

Cells were harvested from the dissolvable microcarriers after 5 to 7 days of expansion. First, a "harvest solution" was prepared in order to fully dissolve the microcarriers and release the attached cells. We recommend adding 250 mL harvest solution per gram of dry microcarriers. The recommended harvest solution for hMSCs is: 100 U/mL pectinase (MilliporeSigma Cat. No. P2611), 10 mM EDTA (Corning Cat. No. 46-034-CI), 5X TryPLE™ Select (Thermo Fisher Cat. No. A12177-01) diluted in DPBS (Corning Cat. No. 21-031-CM). The harvest solution was filter-sterilized (Corning Cat. No. 430767) and pre-warmed to 37°C prior to use.

To begin the harvest process, the 1L GSF was removed from the stir platform and allowed to incubate for 3-5 minutes in order to allow the microcarriers to settle. Next, the spent media was removed and the microcarriers were washed with 200-300 mL DPBS (Corning Cat. No. 21-031-CM). The microcarriers were allowed to settle after the addition of DPBS and the supernatant was then removed. To dissolve the microcarriers, 150 mL of harvest solution was added to the 1L GSF and the flask was incubated on a stir platform for 10-15 minutes at 32 rpm. Once a single-cell suspension was observed, the cells were centrifuged at 200 xg for 5 minutes in a 250 mL centrifuge tube (Corning Cat. No 430776) and then resuspended in CryoStor® CS5 for cryopreservation.

Cell Characterization

To demonstrate that the hMSCs maintained multipotency after expansion on Corning dissolvable microcarriers, the harvested hMSCs were differentiated into adipocytes, chondrocytes, and osteocytes. First, the cells were thawed and washed in culture medium to remove the freezing medium. For adipocyte and osteocyte differentiation, the cells were re-seeded in Corning CellBIND surface 6-well plates (Corning Cat. No. 3335). For chondrocyte differentiation, the cells were re-seeded in Tissue Culture (TC)-treated 6-well plates (Corning Cat. No. 3506). The cells were differentiated according to manufacturer's recommendations (Thermo Fisher Cat. Nos. A10070-01, A10072-01, and A10071-01). After 2-3 weeks, differentiated cells were fixed in 2% paraformaldehyde and stained for characteristic markers of differentiation: adipocytes (Oil Red O; MilliporeSigma Cat. No. O1516-250ML), osteocytes (Alizarin Red; MilliporeSigma Cat. No. TMS-008-C), and chondrocytes (Alcian Blue; MilliporeSigma Cat. No. TMS-010-C).

For quantitative analysis of cell phenotype, cells were thawed and immunostained for positive and negative surface markers. Positive markers included CD73 (BD Biosciences Cat. No. 550256), CD90 (BD Biosciences Cat. No. 555593), CD105 (BD Biosciences Cat. No. 555690). Negative markers included CD14 (MilliporeSigma Cat. No. MAB1219) and CD34 (BD Biosciences Cat. No. 555820). Cells were replated overnight on T-175 flasks (CellBIND surface, coated with Corning Synthemax™ II) to acquire CD105 data. Flow cytometry was used to quantify the percentage of viable cells expressing each marker. Briefly, cells were

diluted to $1x10^6$ cells/mL in 1X DPBS (Corning Cat. No. 21-031) supplemented with 10% heat-inactivated serum (Corning Cat. No. 35-011-CV) (blocking buffer) and 1:1000 dilution of propidium iodide (MilliporeSigma Cat. No. P4864). The cells were then incubated in the dark for 20 minutes at room temperature. Cells were washed twice with 1X DPBS, resuspended in blocking buffer at $0.5x10^6$ cells/50 µL, and incubated with primary antibodies (10 µg/mL) or corresponding IgG1 isotype control (BD Biosciences Cat. No. 554121) for 30 minutes at 4°C in the dark. Cells were washed in staining buffer containing BSA (BD Biosciences Cat. No. 554657) and then incubated with secondary antibody (1:1000 dilution of Alexa Fluor® 488 goat anti-mouse IgG1 [Thermo Fisher Cat. No. A11001]) for 30 minutes at 4°C in the dark. Stained cells were washed once and resuspended in staining buffer for processing on a BD FACSCalibur™ flow cytometer. During acquisition, 30,000 events/sample were collected and analyzed using the BD CellQuest™ Pro software using dot plot analysis with double gating for FSC/SSC and FL3-PI- vs. PI+, live vs. dead cells.

For analysis of cell karyotype, cells were submitted as live cultures for G-banding karyotype analysis (WiCell Research Institute, Cytogenetics Laboratory).

Results

Attachment of hMSCs on Corning® Synthemax™ II-coated Dissolvable Microcarriers

Cell attachment to microcarriers was monitored by quantifying the number of unattached cells in the culture medium for up to 24 hours after cell seeding. Figure 1 shows images of the microcarrier cultures at the time of cell seeding (0 hours) and 24 hours post-cell seeding. By the end of the attachment phase, nearly all of the cells were attached to the microcarriers resulting in an attachment efficiency of 80% to 90%. Importantly, the cells demonstrated uniform attachment to the microcarriers with minimal bare microcarriers observed. Additionally, the cells exhibited the expected cell morphology after the cell attachment phase, which suggests that the microcarrier surface and cell attachment conditions were appropriate.

Expansion of hMSCs on Corning Synthemax II-coated Dissolvable Microcarriers

Cells were expanded on Corning dissolvable microcarriers for 7 days with half volume media exchanges every 2-3 days. Figure 2 shows microscope images of the microcarrier culture during the expansion phase. A uniform distribution of cells was observed for the first 2-3 days of the culture with the cells continuing to demonstrate the expected cell morphology. At the latter phase of the culture period, the cells achieved confluency with the concurrent appearance of microcarrier aggregates. The mixing speed was increased daily during the expansion phase in an attempt to minimize aggregation of the microcarriers; however, a more aggressive mixing strategy may be required to further reduce microcarrier aggregation. Microcarrier aggregation has previously been reported for hMSCs cultured in serum-free medium on other microcarriers[4].

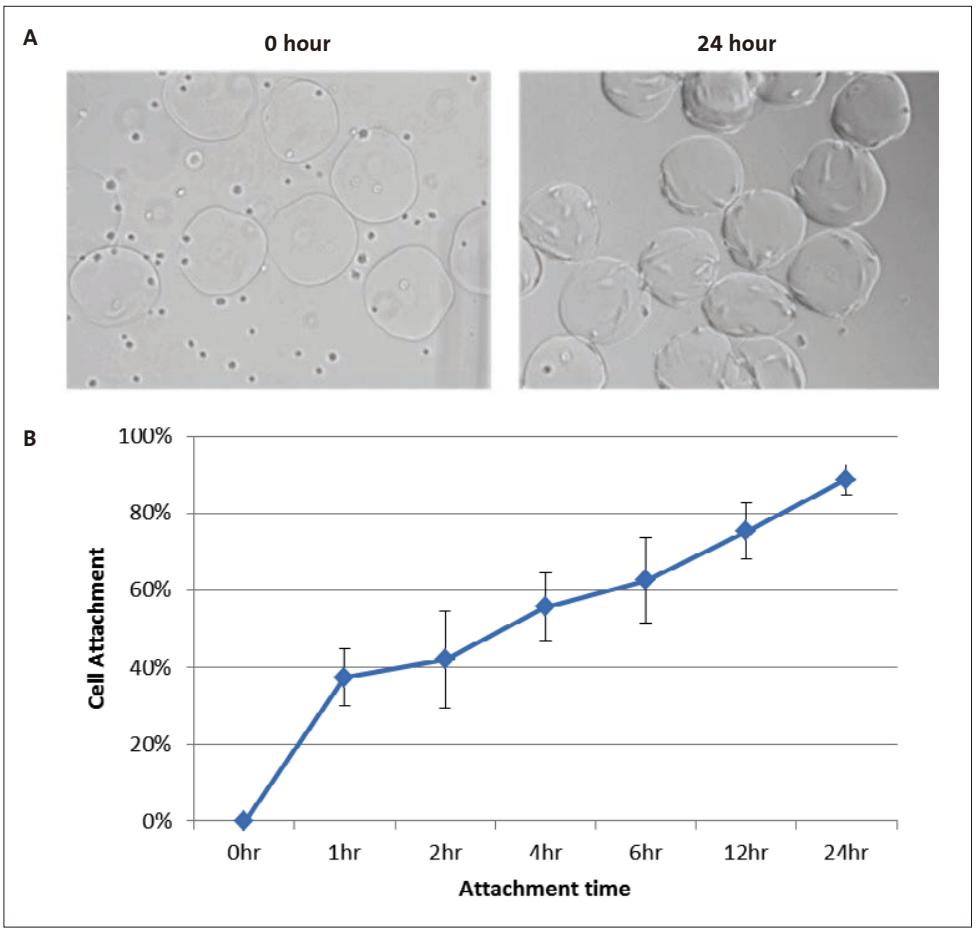


Figure 1. Demonstration of hMSC attachment to Corning Synthemax II-coated dissolvable microcarriers. Panel A: Representative microscope images of hMSCs and Synthemax II-coated dissolvable microcarriers during the cell attachment phase at 0 hours (left panel) and 24 hours (right panel). 10X magnification. Panel B: Cell attachment efficiency for hMSCs on Synthemax II-coated dissolvable microcarriers (n=3). Cell attachment efficiency was determined by quantifying the number of unattached cells in the culture.

Microcarrier Dissolution and Harvest of hMSCs

Cells were harvested from 1L GSFs on days 5, 6, and 7. Figure 3 shows that complete microcarrier dissolution was observed 2-4 minutes after addition of the harvest solution. The harvest phase was allowed to continue for 10-20 minutes in order to generate a single-cell suspension. The total harvest time is dependent upon the level of confluency of the microcarrier culture. Less time may be required for subconfluent microcarriers, whereas longer times may be required for confluent microcarriers.

Figure 4 shows the overall cell yield for each culture following microcarrier dissolution and cell release. On day 5, cell concentrations of 25,000 cells/cm^2 were achieved corresponding to 4-fold expansion. By day 7, cell concentrations routinely exceeded 40,000 cells/cm^2 (>7-fold expansion) with high viability (>90%). Due to the ability to completely dissolve the microcarriers, cell recovery can be significantly improved compared to other commercially available microcarriers.

Characterization of hMSCs Harvested from Corning® Dissolvable Microcarriers

hMSCs harvested from the dissolvable microcarriers were fully characterized for cell marker expression, karyotype, and the ability to differentiate into adipocyte, chondrocyte, and osteocyte cell

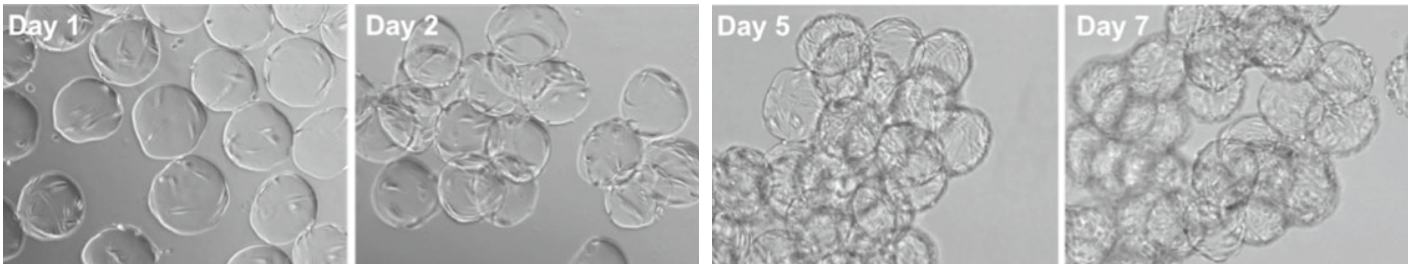


Figure 2. Images of cell expansion on Corning Synthemax™ II-coated dissolvable microcarriers. Representative microscope images of hMSCs on Synthemax II-coated dissolvable microcarriers on days 1, 2, 5, and 7 (10X magnification).

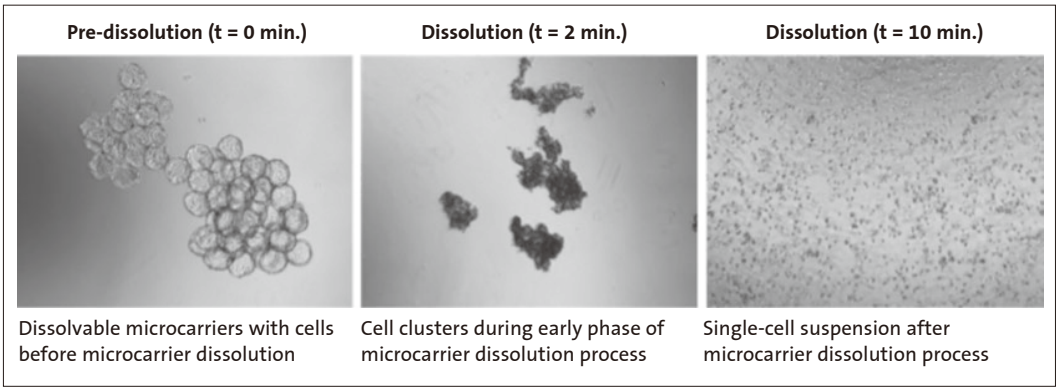


Figure 3. Microcarrier dissolution and cell release. Representative microscope images at different time points during the cell harvest phase (4X magnification). Microcarriers were dissolved in a 1L glass spinner flask following the addition of a harvest solution consisting of pectinase, EDTA, and TrypLE™. The microcarrier culture was mixed at 32 rpm during the harvest phase.

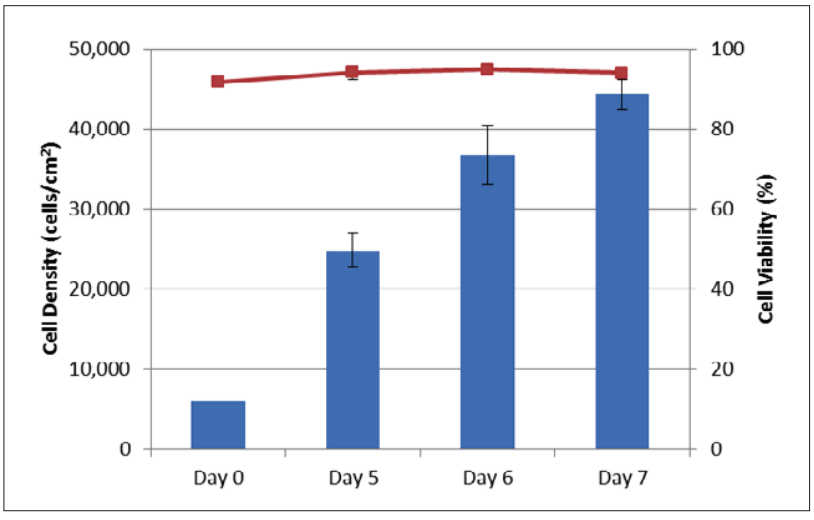


Figure 4. Quantitation of cell yield and cell viability following microcarrier dissolution and cell release. The entire microcarrier culture was harvested from individual 1L glass spinner flasks on days 5, 6, and 7. Following microcarrier dissolution and cell release, cells were quantified on a Vi-CELL™ automated cell counter. Blue bars represent cell concentration (cells/cm^2); red line represents cell viability following cell harvest (n = 2, Days 5 and 6; n = 3, Day 7).

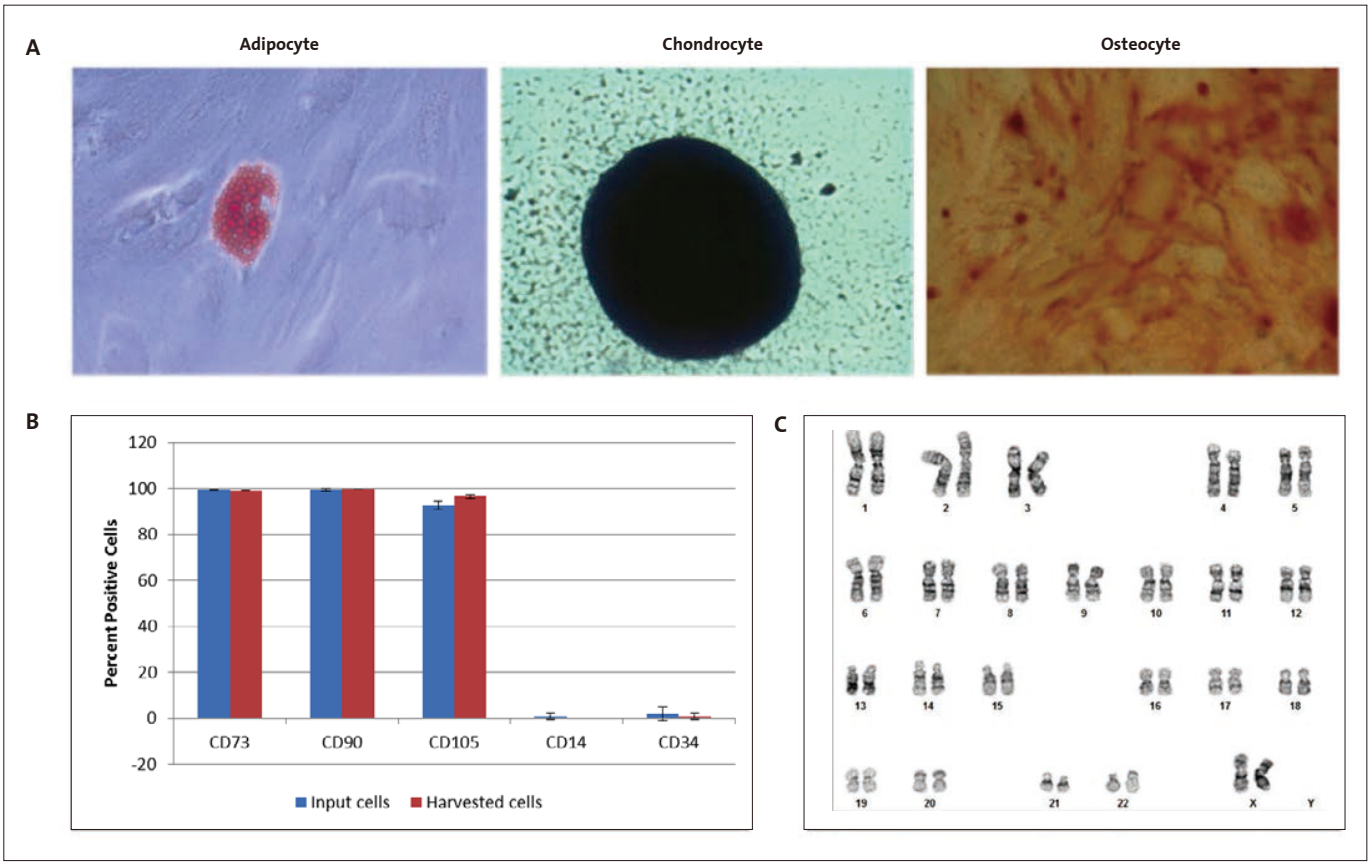


Figure 5. Characterization of hMSCs harvested from Corning Synthemax II-coated dissolvable microcarriers. Cells harvested from dissolvable microcarriers were characterized for 1) ability to differentiate into adipocytes, chondrocytes, and osteocytes; 2) expression of cell surface markers; and 3) genetic stability. Panel A: Confirmation of terminal differentiation of adipocyte, chondrocyte, and osteocyte cell lineages following incubation with the appropriate differentiation medium for 14-21 days. Cell differentiation was confirmed by staining with Oil Red O (adipocyte), Alcian Blue (chondrocyte), and Alizarin Red S (osteocyte). Adipocyte (20X magnification), chondrocyte, and osteocyte (2.5X magnification). Panel B: Representative flow cytometry data for specific cell surface markers (n = 2). Input cells represent cells that were used to seed the dissolvable microcarriers. Panel C: Demonstration of normal karyotype for hMSCs harvested from dissolvable microcarriers. Live cultures were submitted for G-banded karyotype analysis.

lineages (Figure 5). Terminal differentiation into adipocyte, chondrocyte, and osteocyte lineages was confirmed using traditional staining methods for characteristic markers. Flow cytometry confirmed the expression of the positive surface markers CD73, CD90, and CD105 and the absence of CD14 and CD34. Chromosome analysis confirmed that the cells maintained the normal karyotype. The characterization results demonstrate that hMSCs expanded and harvested from dissolvable microcarriers maintain their phenotype, multipotency, and genetic stability.

Summary/Conclusions

- Novel cell culture platforms are needed to meet the expected future demand of hMSCs that is being driven by the emergence of regenerative medicine and cell-based therapy. Existing cell culture platforms have limitations that hamper their ability to generate sufficient quantities of therapeutically active cells using well-controlled processes. Corning® dissolvable microcarriers provide a scalable solution for the large-scale expansion and harvest of functional hMSCs by enabling simplified downstream processing and high yield cell recovery.
- A user-friendly protocol was developed to efficiently expand hMSCs on Corning Synthemax™ II-coated dissolvable microcarriers in a serum-free medium.
- Complete dissolution of the microcarriers was observed during the harvest phase resulting in high recovery of functional hMSCs.
- hMSCs harvested from dissolvable microcarriers maintained multipotency and demonstrated the expected phenotype.
- Corning Synthemax™ II-coated dissolvable microcarriers provide an ideal platform for the scale-up and harvest of functional hMSCs for bioprocess applications.

References

1. T. Squillaro, G. Peluso, and U Galderisi, Cell Transplant 25 (5):829-848 (2016).
2. A. Trounson and C. McDonald, Cell Stem Cell 17:11-22 (2015).
3. B. Dodson and A. Levine, BMC Biotechnology 15:70 (2015).
4. T. Heathman, et al., Biotechnology and Bioengineering 112 (8):1696-1707 (2015).

康宁生命科学 (吴江) 有限公司
中国江苏吴江经济开发区
庞金路1801号T03/17厂房
邮编: 215200
电话: +86-512-6319-6555
传真: +86-512-6319-6502

上海办事处
中国上海市漕河泾高新技术开发区
桂箐路111号立明大厦6楼
邮编: 200233
电话: +86-21-3338-4338
传真: +86-21-3338-4300

北京办事处
中国北京市建国门外大街1号
国贸大厦1座2716室
邮编: 100004
电话: +86-10-6539-0888
传真: +86-10-6505-5077

CORNING | FALCON AXYGEN PYREX

400-600-0207
ScientificSupportAP@corning.com
www.corning.com/lifesciences/china

www.cls-china.cn
www.cellculturesuccess.com