Microfluidic-Engineered Preparation of Poly-L-lactic Acid Porous Microspheres as Cell Scaffolds

Zhaofan HU [1, 2], Yage LIU [1, 2], Perumal RAMESH KANNAN [1, 2], Jingjing ZHANG [1, 2], Xiangdong KONG [1, 2], Ruibo ZHAO [1, 2, 3] *

[1] *Institute of Smart Biomaterials, School of Materials Science and Engineering, Zhejiang Sci-Tech University, Hangzhou 310018, Zhejiang, China*
[2] *Zhejiang-Mauritius Joint Research Center for Biomaterials and Tissue Engineering, Hangzhou 310018, Zhejiang, China*
[3] *Foundation of Zhejiang Sci-Tech University Shengzhou Innovation Research Institute, Shengzhou 312451, Zhejiang, China*

http://doi.org/10.5755/j02.ms.38517

Received 19 August 2024; accepted 2 December 2024

Regenerative medicine has emerged as a promising field to address tissue damage and organ failure, with porous microspheres playing a crucial role as cell carriers in tissue engineering applications. However, conventional fabrication methods often result in heterogeneous size distributions and poorly controlled pore structures, limiting their effectiveness. This study leverages microfluidic technology to overcome these challenges and develop uniform poly (L-lactic acid) (PLLA) porous microspheres. We comprehensively evaluate their physical and biological properties, including morphology, size distribution, pore structure, and biocompatibility. Through long-term culture experiments, we investigated the microspheres' capacity to support cell growth and proliferation, demonstrating their effectiveness as cell scaffolds. Our research provides valuable insights into the potential of microfluidic-produced PLLA porous microspheres and its further translational medicine.
Keywords: tissue engineering, porous microspheres, microfluidic, PLLA, cell scaffolds.

1. INTRODUCTION

Regenerative medicine has emerged as a groundbreaking field in modern healthcare, offering promising solutions to address the growing challenges of tissue damage, organ failure, and degenerative diseases [1, 2]. The core of regenerative medicine is tissue engineering, which aims to restore, maintain, or enhance tissue function by combining cells and biomaterials [3]. Central to the success of tissue engineering strategies is the development of appropriate scaffolds that provide a suitable microenvironment for cell growth, proliferation, and differentiation [4 – 6].

Porous microspheres have gained significant attention among various scaffold designs due to their unique advantages in tissue engineering applications, mainly as cell carriers [7 – 9]. These three-dimensional structures have several benefits, including their high surface-to-volume ratio, and improved cell attachment and proliferation, which makes them very attractive for minimally invasive cell delivery therapies [10 – 12]. The porous nature of these microspheres provides an ideal environment for cell adhesion and growth, while their spherical shape allows for uniform cell distribution and easy handling [7, 9, 13]. Moreover, porous microspheres promote cell-to-cell interactions and the formation of three-dimensional tissue-like structures, which are crucial for many tissue engineering applications [7, 8]. Poly (L-lactic acid) (PLLA) has emerged as a promising candidate for optimal materials to fabricate these porous microspheres. PLLA is a biocompatible polymer with excellent mechanical properties and processability, making it highly suitable for various tissue engineering applications [14, 15]. Its versatility allows for the creation of porous microspheres with controlled physical properties to meet the requirements of multiple cell types and tissue engineering strategies [16, 17]. Additionally, they are widely employed as a source of chirality for self-organizing low molar mass [18 – 20] and macromolecular systems [21 – 23], thus demonstrating their broader significance and versatility across various scientific and industrial areas.

Conventional methods for preparing porous microspheres, such as emulsion solvent evaporation and phase separation, often result in heterogeneous size distributions and poorly controlled pore structures [7, 24]. These limitations can significantly impact the performance of microspheres in biological applications, where uniform size and pore structure are crucial for consistent cell behavior and distribution [25]. To overcome these challenges, microfluidic technology has emerged as a promising approach for particle production. This technology offers precise particle size and morphology control, improved reproducibility, and reduced batch-to-batch variations [24, 26, 27]. These advantages make microfluidic approaches particularly attractive for producing PLLA porous microspheres with tailored properties for tissue engineering applications.

This study uses microfluidic technology to develop uniform PLLA porous microspheres and comprehensively evaluate their potential as cell scaffolds and delivery

* Corresponding author: R. Zhao
E-mail: *rzhao@zstu.edu.cn*

vehicles. Our approach includes a thorough characterization of the microspheres' physical and biological properties, such as morphology, size distribution, pore structure and biocompatibility. We examine the microspheres' capacity to promote cell growth and proliferation through extended culture studies, proving their efficacy as cell scaffolds. This comprehensive evaluation aims to provide valuable insights into the potential of these microfluidic-produced microspheres for various applications in tissue engineering and regenerative medicine.

2. EXPERIMENTAL

To prepare PLLA porous microspheres, we constructed a microfluidic platform using an 18G/25G coaxial needle. A 1 % PVA solution was used for both the continuous phase and the collection solution. We dissolved PLLA (2.5 %, w/v) in dichloromethane and 0.1 g ammonium bicarbonate in 10 ml aqueous solution. The PLLA and ammonium bicarbonate were mixed in a 3:1 ratio and homogenized in the dispersed phase at 5000 rpm for 2 min. The flow rate of the dispersed phase was set at 0.1 mL/min and the continuous phase at 2 mL/min. Shearing on a microfluidic platform formed Homogeneous droplets, and mechanical stirring evaporated the solvent overnight. Ammonium bicarbonate decomposed to form a pore structure. After washing and lyophilization, we obtained porous microspheres (Fig. 1), and they were named PLLA PMs.

We used a stereomicroscope (NSZ-608T, China) and an optical microscope (Leica, Germany) to observe the shape of the microspheres. The particle size of the microspheres was then measured based on the microscope imaging results (n = 100). We subsequently observed the morphology of the microspheres using SEM (Zeiss, USA) and measured their pore size. To assess the cytotoxicity, the microspheres were sterilized by UV light for 2 h and subsequently immersed in αMEM (Cytiva, China) containing serum and antibiotics for 24 hours. The extract was filtered and subsequently diluted to create six concentration gradients, with concentrations of 4, 2.5, 1, 0.5, 0.25, and 0.125 mg/mL, respectively. MC3T3-E1 cells were seeded in 96-well plates, and the medium was replaced with microsphere extract after 24 hours. The cells were incubated for another 24 hours. After adding the CCK-8 solution (Beyotime, China), we measured the absorbance at 450 nm to assess cell viability after 1 h of incubation.

We sterilized PLLA PMs with alcohol and co-cultured them with MC3T3-E1 cells in anti-adhesion treated well plates. The solution was replaced every two days, and the live and dead cell dyes were introduced after 3, 5, and 10 days of incubation. We observe the cell viability using confocal microscopy (Nikon, Japan) after a PBS wash, with green indicating live cells and red indicating dead cells.

4. RESULTS AND DISCUSSION

We prepared PLLA PMs by combining the microfluidic technique with solvent volatilization. The microfluidic technique made droplets that were about 500 μm in diameter and had a very uniform size and shape because they had excellent monodispersity and spherical structure as seen under Stereomicroscope (Fig. 2 a). The microscope images further confirmed the homogeneity of the microspheres (Fig. 2 b). Also revealed that the microspheres possessed a certain degree of light transmittance, which implied the possible existence of a porous structure within them. SEM images provided a high-resolution view of the microsphere surface structure (Fig. 2 c), demonstrating the uniformly distributed porous structure. These pores vary in size and form a honeycomb-like surface morphology. When using porous microspheres as cell carriers, the particle size plays a crucial role. A reduction in particle size increases the microsphere's surface area-to-volume ratio, facilitating cell-to-microsphere contact and material exchange.

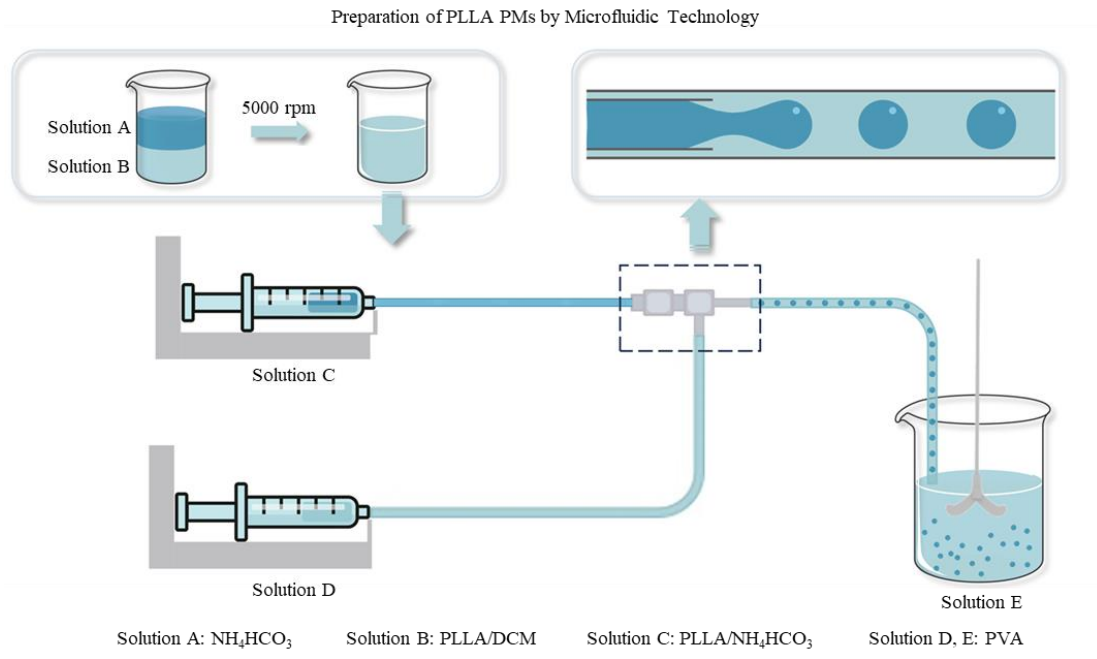
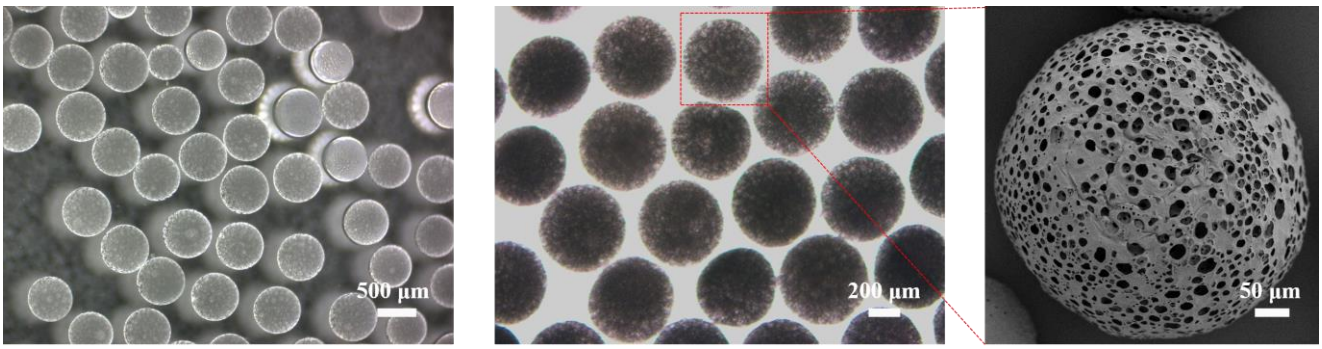


Fig. 1. Schematic illustration for the preparation of PLLA PM



a b c

Fig. 2. PLLA PMs images: a – stereoscopic; b – optical microscopy; c – SEM

However, a tiny particle size may increase the risk of cellular phagocytosis. In contrast, large particle size is more conducive to maintaining the growth of cell populations and forming larger tissue structures [28]. The particle size measurements showed that the porous microspheres were predominantly distributed between 400 and 550 µm, with an average size of 469.61 µm. Compared to conventional microsphere preparation methods, microfluidic technology produces microspheres with a more uniform size distribution. Additionally, pore size significantly influences various cellular processes, including adhesion, proliferation, migration, and the transport of nutrients and metabolic waste [29]. Porous microspheres with the appropriate pore size are critical for promoting cell migration and growth as a cell culture carrier [30]. The pore size measurements indicated that the microsphere pores were predominantly distributed between 10 and 40 µm, with an average pore size of 18.04 µm (Fig. 3 b). This range is sufficient to meet the requirements for cell migration, nutrient transport, and gas exchange within the pores.

Ideal biomaterials should demonstrate excellent biocompatibility. We used the CCK-8 assay to assess cell viability by measuring cell metabolic activity and determining whether the prepared PLLA PMs meet this essential criterion. MC3T3-E1 cells were treated with six different concentrations of PLLA PMs extracts, and the cytotoxicity of the materials at these concentrations was evaluated (Fig. 4).

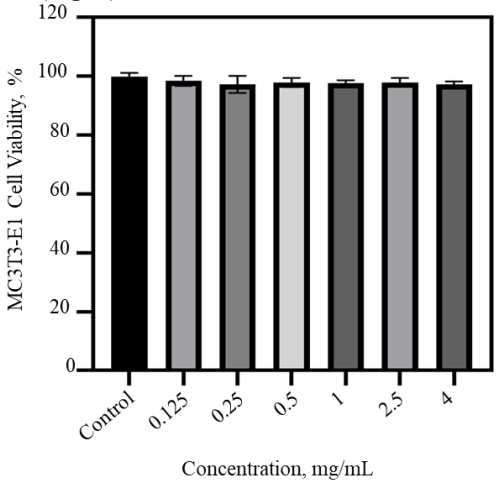
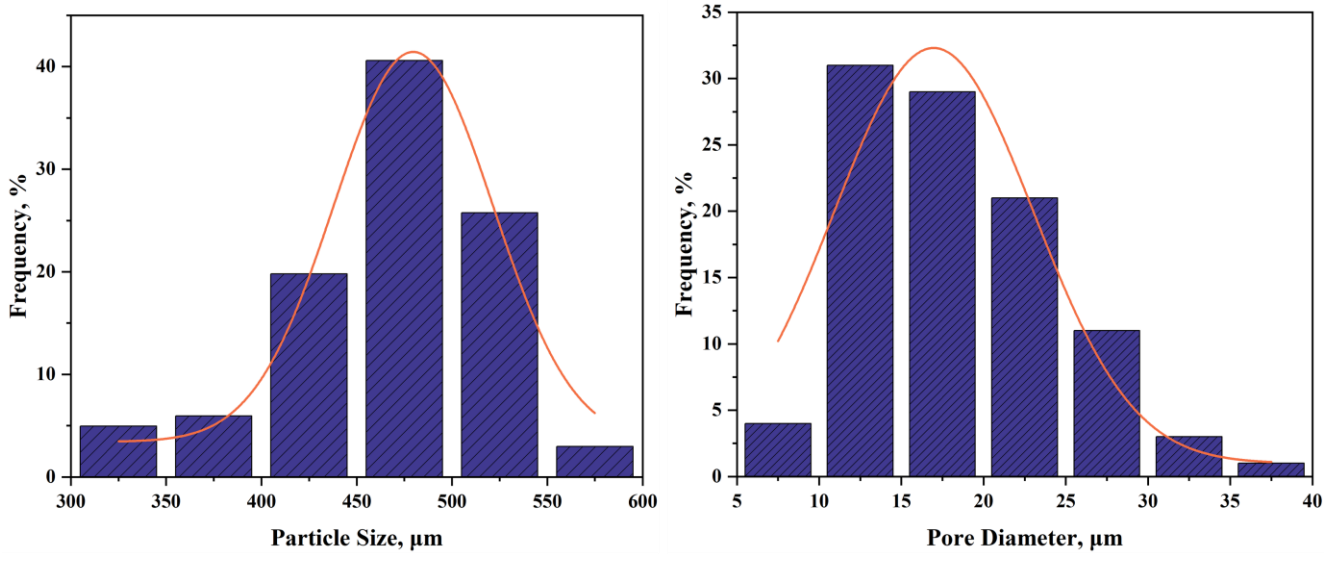


Fig. 4. Cell viability of MC3T3-E1 on PLLA PMs at various concentrations (CCK-8 assay)



a b

Fig. 3. Distribution of PLLA PMs: a – particle size; b – pore size

According to the microscopic imaging results (Fig. 5 a), on day 3 of co-culture, the cells were mostly present as single cells on the microspheres, with very few clustered aggregates. The microspheres also tended to be monodisperse, suggesting that the PMs provide a favorable attachment site for the cells and promote initial cell adhesion. On day 5, the cells formed clusters and interconnected with the microspheres. On day 10, most of the cells had attached to the microspheres in clusters, forming a densely connected cell-material composite. The live-dead staining revealed that most of the cells adhered to the surface of the microspheres on day 3 of the co-culture (Fig. 5 b, c). Initially, we observed fewer cells in the interior of the microspheres. However, as the co-culture period progressed, the number of cells increased, eventually covering the whole surface of the microspheres by day 10. Almost no PI (propidium iodide)-labeled dead cells were visible, and the green fluorescence indicated that most of the cells were alive. This shows that the porous microspheres, served as suitable three-dimensional porous scaffolds, are biocompatible and could provide an alternative microenvironment for cell growth. Besides, PLLA PMs can promote the initial adhesion and proliferation of cells and support the formation of a stable three-dimensional network structure. Meanwhile, PLLA PMs exhibited excellent biocompatibility and maintained high cell viability and low mortality.

4. CONCLUSIONS

In this study, we prepared uniform PLLA PMs using microfluidic technology and comprehensively evaluated their potential as cell scaffolds and delivery vehicles. We confirmed the superiority of these microspheres for tissue engineering applications through in-depth characterization of their physical and biological properties, including morphology, particle size distribution, pore structure, and biocompatibility. Long-term culture experiments further demonstrate the ability of microspheres to support cell growth and proliferation. This study provides an alternative insight into regenerative medicine and offers new opportunities to address health challenges such as tissue damage, organ failure, and degenerative diseases.

Acknowledgments

The financial support for this work was provided by the key project of the Natural Science Foundation of Zhejiang Province (LZ24E020002), Foundation of Zhejiang Sci-Tech University Shengzhou Innovation Research Institute (SYY2024C000012), and the Key Research & Development Program of Zhejiang Province (2024C03019, 2019C04020), and the discipline construction funding of biomedical materials in ZSTU.

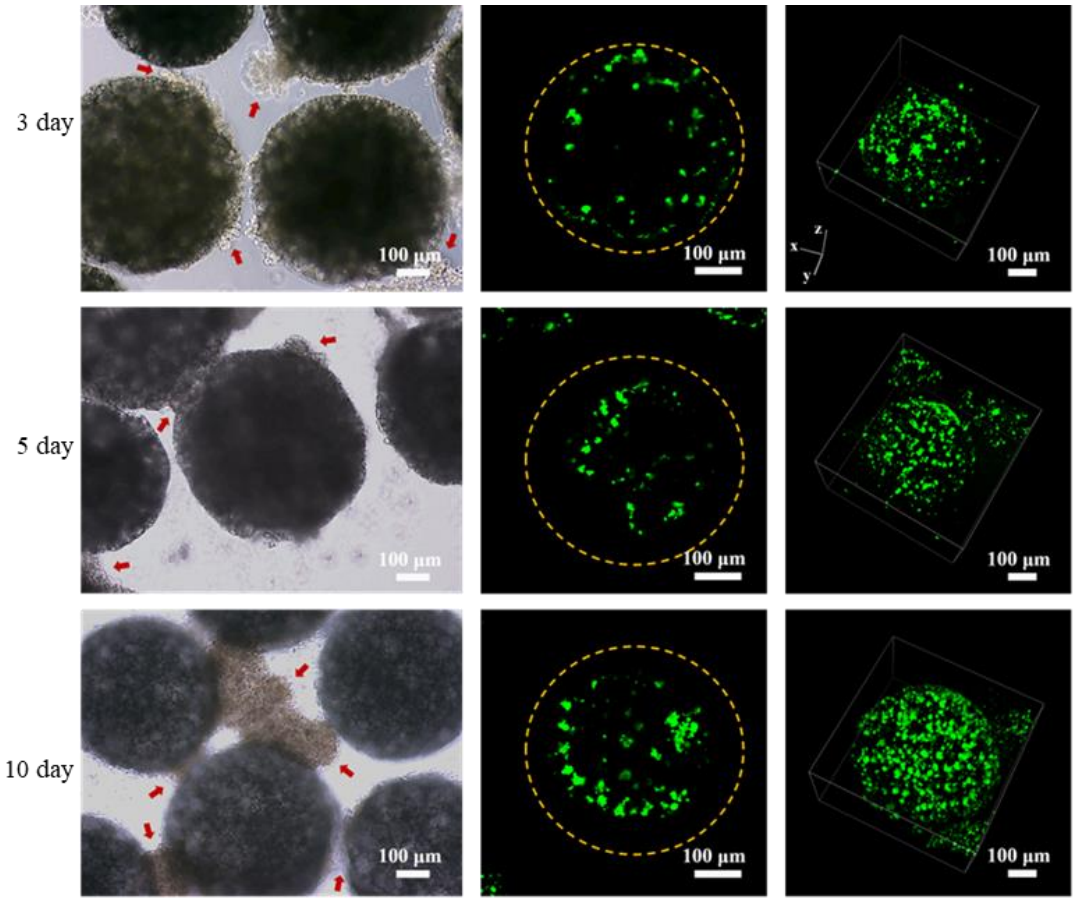


Fig. 5. PLLA PMs and MC3T3-E1 cells co-culture: a – microscopic images at 3, 5, and 10 days; b – live-dead staining; c – 3D confocal microscopy

REFERENCES

1. **Arjmand, B., Rabbani, Z., Soveyzi, F., Tayanloo-Beik, A., Rezaei-Tavirani, M., Biglar, M., Adibi, H., Larijani, B.** Advancement of Organoid Technology in Regenerative Medicine *Regenerative Engineering and Translational Medicine* 9 (1) 2023: pp. 83 – 96. https://doi.org/10.1007/s40883-022-00271-0
2. **Park, S., Rahaman, K.A., Kim, Y.C., Jeon, H., Han, H. S.** Fostering Tissue Engineering and Regenerative Medicine to Treat Musculoskeletal Disorders in Bone and Muscle *Bioactive Materials* 40 2024: pp. 345 – 365. https://doi.org/10.1016/j.bioactmat.2024.06.022.
3. **Liu, S., Yu, J.M., Gan, Y.C., Qiu, X.Z., Gao, Z.C., Wang, H., Chen, S.X., Xiong, Y., Liu, G.H., Lin, S.E., McCarthy, A., John, J.V., Wei, D.X., Hou, H.H.** Biomimetic Natural Biomaterials for Tissue Engineering and Regenerative Medicine: New Biosynthesis Methods, Recent Advances, And Emerging Applications *Military Medical Research* 10 (1) 2023: pp. 16. https://doi.org/10.1186/s40779-023-00448-w.
4. **Zhou, J., Xiong, S., Liu, M., Yang, H., Wei, P., Yi, F., Ouyang, M., Xi, H., Long, Z., Liu, Y., Li, J., Ding, L., Xiong, L.** Study on the Influence Of Scaffold Morphology and Structure on Osteogenic Performance *Frontiers in Bioengineering and Biotechnology* 11 2023: pp. 1127162. https://doi.org/10.3389/fbioe.2023.1127162.
5. **Krishani, M., Shin, W.Y., Suhaimi, H., Sambudi, N.S.** Development of Scaffolds from Bio-Based Natural Materials for Tissue Regeneration Applications: A Review *Gels* 9 (2) 2023: pp. 100. https://www.mdpi.com/2310-2861/9/2/100.
6. **Wang, H., Chang, X., Ma, Q., Sun, B., Li, H., Zhou, J., Hu, Y., Yang, X., Li, J., Chen, X., Song, J.** Bioinspired Drug-Delivery System Emulating the Natural Bone Healing Cascade for Diabetic Periodontal Bone Regeneration *Bioactive Materials* 21 2023: pp. 324 – 339. https://doi.org/10.1016/j.bioactmat.2022.08.029.
7. **Li, Q., Chang, B., Dong, H., Liu, X.** Functional Microspheres for Tissue Regeneration *Bioactive Materials* 25 2023: pp. 485 – 499. https://doi.org/10.1016/j.bioactmat.2022.07.025.
8. **Duan, C., Yu, M., Hu, C., Xia, H., Kankala, R.K.** Polymeric Microcarriers for Minimally-Invasive Cell Delivery *Frontiers in Bioengineering and Biotechnology* 11 2023: pp. 1076179. https://doi/org10.3389/fbioe.2023.1076179.
9. **Li, Y., Liu, S., Zhang, J., Wang, Y., Lu, H., Zhang, Y., Song, G., Niu, F., Shen, Y., Midgley, A.C., Li, W., Kong, D., Zhu, M.** Elastic Porous Microspheres/Extracellular Matrix Hydrogel Injectable Composites Releasing Dual Bio-Factors Enable Tissue Regeneration *Nature Communications* 15 (1) 2024: pp. 1377. https://doi.org/10.1038/s41467-024-45764-4.
10. **Bai, L., Zhang, X., Han, Z., Yang, X., Hao, Y.** Injectable Porous Microspheres for Articular Cartilage Regeneration Through in Situ Stem Cell Recruitment and Macrophage Polarization *Acta Biomaterialia* 2024: pp. 429 – 440. https://doi.org/10.1016/j.actbio.2024.07.007.
11. **Bai, L., Han, Q., Han, Z., Zhang, X., Zhao, J., Ruan, H., Wang, J., Lin, F., Cui, W., Yang, X., Hao, Y.** Stem Cells Expansion Vector via Bioadhesive Porous Microspheres for Accelerating Articular Cartilage Regeneration *Advanced Healthcare Materials* 13 (3) 2024: pp. 2302327. https://doi.org/10.1002/adhm.202302327.
12. **Zhu, Y., Yi, D., Wang, J., Zhang, Y., Li, M., Ma, J., Ji, Y., Peng, J., Wang, Y., Luo, Y.** Harnessing Three-Dimensional Porous Chitosan Microsphere Embedded with Adipose-Derived Stem Cells to Promote Nerve Regeneration *Stem Cell Research & Therapy* 15 (1) 2024: pp. 158. https://doi.org/10.1186/s13287-024-03753-w.
13. **Shi, X., Cui, L., Sun, H., Jiang, N., Heng, L., Zhuang, X., Gan, Z., Chen, X.** Promoting Cell Growth on Porous PLA Microspheres Through Simple Degradation Methods *Polymer Degradation and Stability* 161 2019: pp. 319 – 325. https://doi.org/10.1016/j.polymdegradstab.2019.01.003.
14. **Wang, P., Wang, M., Wei, X., Xie, L., Tian, L., Yang, Z., Zhou, Z., Chen, H.** In vitro and In Vivo Degradation Profile, Biocompatibility of Poly-L-Lactic Acid Porous Microspheres *International Journal of Biological Macromolecules* 272 2024: pp. 132876. https://doi.org/10.1016/j.ijbiomac.2024.132876.
15. **Li, B., Zu, M., Jiang, A., Cao, Y., Wu, J., Shahbazi, M.A., Shi, X., Reis, R.L., Kundu, S.C., Xiao, B.** Magnetic Natural Lipid Nanoparticles for Oral Treatment of Colorectal Cancer Through Potentiated Antitumor Immunity and Microbiota Metabolite Regulation *Biomaterials* 307 2024: pp. 122530. https://doi.org/10.1016/j.biomaterials.2024.122530.
16. **Polak, M., Berniak, K., Szewczyk, P.K., Karbowniczek, J.E., Marzec, M.M., Stachewicz, U.** PLLA Scaffolds with Controlled Surface Potential and Piezoelectricity for Enhancing Cell Adhesion in Tissue Engineering *Applied Surface Science* 621 2023: pp. 156835. https://doi.org/10.1016/j.apsusc.2023.156835.
17. **Castañeda-Rodríguez, S., González-Torres, M., Ribas-Aparicio, R.M., Del Prado-Audelo, M.L., Leyva-Gómez, G., Gürer, E.S., Sharifi-Rad, J.** Recent Advances in Modified Poly (Lactic Acid) as Tissue Engineering Materials *Journal of Biological Engineering* 17 (1) 2022: pp. 21. https://doi.org/10.1016/j.mtadv.2022.100205
18. **Bubnov, A., Novotná, V., Hamplová, V., Kašpar, M., Glogarová, M.** Effect of Multilactate Chiral Part of Liquid Crystalline Molecule on Mesomorphic Behavior *Journal of Molecular Structure* 892 (1) 2008: pp. 151 – 157. https://doi.org/10.1016/j.molstruc.2008.05.016.
19. **Anna, P., Alexej, B., Damian, P., Jiří, S., Michal, K.** The Effect of the Length of Terminal N-Alkyl Carboxylate Chain on Self-Assembling and Photosensitive Properties of Chiral Lactic Acid Derivatives *Journal of Molecular Liquids* 275 2019: pp. 829 – 838. https://doi.org/10.1016/j.molliq.2018.11.058.
20. **Bubnov, A., Cigl, M., Mironov, S., Otruba, M., Penkov, D., Pacherová, O., Bohmová, Z., Hamplová, V.** The Correlations Between the Molecular Core Structure and Mesomorphic Behaviour for Chiral Liquid Crystals with Several (S)-Lactate Groups *Journal of Molecular Liquids* 387 2023: pp. 122590. https://doi.org/10.1016/j.molliq.2023.122590.
21. **Petrova, I., Gaj, A., Pochiecha, D., Shcherbina, M., Makarova, N.N., Bubnov, A.** Design and Self-Assembling Behaviour of Comb-Like Stereoregular Cyclolinear Methylsiloxane Copolymers with Chiral Lactate Groups *Liquid Crystals* 46 (1) 2019: pp. 25 – 36. https://doi.org/10.1080/02678292.2018.1461265.
22. **Bubnov, A., Domenici, V., Hamplová, V., Kašpar, M., Zalar, B.** First Liquid Single Crystal Elastomer Containing Lactic Acid Derivative as Chiral Co-Monomer: Synthesis and Properties *Polymer* 52 (20) 2011: pp. 4490 – 4497.

https://doi.org/10.1016/j.polymer.2011.07.046.

23. **Boychuk, A., Shibaev, V., Cigl, M., Pomeisl, K., Hamplová, V., Pociecha, D., Bubnov, A., Bobrovsky, A.** Photo-Orientation Processes in Liquid Crystalline Polymethacrylates with Side Azobenzene Groups Having Lateral Methyl Substituents *Macromolecules* 54 (22) 2021: pp. 10499 – 10509. https://doi.org/10.1021/acs.macromol.1c01637
24. **Shi, Y., Zhang, X., Mu, K., Wang, Y., Jiang, T., Jiang, S., Zhang, S., Du, Y.** Precise Fabrication of Porous Microspheres by Iso-Density Emulsion Combined with Microfluidics *Polymers* 14 (13) 2022: pp. 2687. https://doi.org/10.1016/j.polymdegradstab.2019.01.003.
25. **Long, F., Guo, Y., Zhang, Z., Wang, J., Ren, Y., Cheng, Y., Xu, G.** Recent Progress of Droplet Microfluidic Emulsification Based Synthesis of Functional Microparticles *Global Challenges* 7 (9) 2023: pp. 2300063. https://doi.org/10.1002/gch2.202300063.
26. **Liu, Z., Fontana, F., Python, A., Hirvonen, J.T., Santos, H.A.** Microfluidics for Production of Particles: Mechanism, Methodology, and Applications *Small* 16 (9) 2020: pp. 1904673. https://doi.org/10.1002/smll.201904673.
27. **Bendre, A., Bhat, M.P., Lee, K.H., Altalhi, T., Alruqi, M.A., Kurkuri, M.** Recent Developments in Microfluidic Technology for Synthesis and Toxicity-Efficiency Studies of Biomedical Nanomaterials *Materials Today Advances* 13 2022: pp. 100205. https://doi.org/10.1016/j.mtadv.2022.100205.
28. **Champion, J.A., Walker, A., Mitragotri, S.** Role of Particle Size in Phagocytosis of Polymeric Microspheres *Pharmaceutical Research* 25 (8) 2008: pp. 1815 – 1821. https://doi.org/10.1007/s11095-008-9562-y.
29. **Cai, Z., Jiang, H., Lin, T., Wang, C., Ma, J., Gao, R., Jiang, Y., Zhou, X.** Microspheres in Bone Regeneration: Fabrication, Properties and Applications *Materials Today Advances* 16 2022: pp. 100315. https://doi.org/10.1016/j.mtadv.2022.100315.
30. **Saif, U.R.M., Wu, J., Chen, H., Sun, C., Liu, Y., Xu, S.** Matrix Mechanophysical Factor: Pore Size Governs the Cell Behavior in Cancer *Advances in Physics: X* 8 (1) 2023: pp. 2153624. https://doi.org/10.1080/23746149.2022.2153624.