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聚磷酸酯/聚乙二醇复合水凝胶的构建及性能

张正海, 杨显珠*

华南理工大学医学院, 广州 510006

* 联系人, E-mail: yangxz@scut.edu.cn

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摘要 在水溶性大分子聚磷酸酯二丙烯酸酯(PEEP-DA)与聚乙二醇二丙烯酸酯(PEG-DA)中加入光引发剂I2959, 在紫外光照射下交联形成三维水凝胶体系. 研究结果表明, 与聚乙二醇水凝胶相比, 聚磷酸酯/聚乙二醇复合水凝胶具有更好的生物可降解性. 其中, PEEP-DA/PEG-DA比例为5:25的复合水凝胶植入到小鼠体内28 d后基本完全消失. 而包裹骨髓间充质干细胞(BMSCs)后, 复合水凝胶体系中细胞活性更好; 聚磷酸酯/聚乙二醇复合水凝胶能够显著增强碱性磷酸酶活性, 提高钙沉积. 本研究提供了一种新的聚磷酸酯/聚乙二醇复合水凝胶体系, 在作为细胞支架方面有一定应用潜力.

关键词 聚磷酸酯, 水凝胶, 支架材料, 生物可降解支架

水凝胶支架具有三维交联的亲水网络结构[1], 其有较高的水含量, 具备多孔性、较好的生物相容性; 同时水凝胶体系还具有黏弹性、机械性能等, 广泛用于药物递送[2]、再生医学[3]等生物医学领域. 随着细胞生物技术和3D打印等精密加工技术的发展, 水凝胶体系在再生医学领域的应用越来越广泛[4~6]. 特别是近年来, 由于全球中高年龄人口比例增加, 骨相关疾病已成为一个令人关注的问题. 因此, 通过在骨组织工程支架中加入各种生长因子、药物、基因或者种子细胞, 使其具有生物活性, 用来植入骨缺损部位诱导骨生成[7,8]. 例如陈学思课题组[9]开发了一种独特的聚乳酸-羟基乙酸共聚物/羟基磷灰石/聚丙交酯-苯胺五聚体复合支架, 将骨形态发生蛋白4有效整合到活性骨修复材料中, 实现了可控、快速、有效地修复骨缺损.

聚磷酸酯是一类以磷酸酯键为链接结构的生物可降解高分子, 20世纪80年代, Kaluzynski等人[10]首先合成了系列聚磷酸酯材料. 目前, 聚磷酸酯材料用于药物递送载体和组织工程支架已被广泛报道. 例如, 美国Leong课题组[11]在聚磷酸酯用作质粒DNA的载体方面开展了系统的研究. 国内王均课题组[12~14]合成了多种聚磷酸酯载体系统, 用于疏水性化疗药物以及siRNA的负载及递送; 颜德岳课题组[15~17]在超支化聚磷酸酯的材料制备以及用于小分子药物载体方面开展了系统的研究. 在组织工程领域, 聚磷酸酯材料曾被用作支架材料用于神经导管再生[18~20]. 后来, 研究发现聚磷酸酯材料能诱导骨的生成, 因此这类材料目前被广泛应用于骨组织工程[21].

本研究合成了一种水溶性聚磷酸酯二丙烯酸酯大分子单体(PEEP-DA), 与聚乙二醇二丙烯酸酯(PEG-DA)混合并加入光引发剂后, 与间充质干细胞混合, 可以在紫外光照射下交联形成组织工程支架. 本工作系统研究了该水凝胶支架的结构、生物可降解性、生物相容性以及诱导骨分化等性能.

1 实验

(ⅰ) 合成PEG-DA. 实验所用试剂(纯度≥99.9%)购

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自国药集团化学试剂有限公司. 聚乙二醇均聚物(链接数为77, 34 g, 10.0 mmol, 纯度>97%, Sigma Aldrich, 美国)加入到500 mL的三口烧瓶中, 并加入200 mL的无水四氢呋喃溶液; 待完全溶解后, 将整个体系置于–5°C反应浴中, 并通过恒压滴液漏斗逐滴加入三乙胺(1.52 g, 15.0 mmol)和丙烯酰氯(1.36 g, 15 mmol)的四氢呋喃溶液(40 mL). 在–5°C反应浴中, 继续反应24 h后, 过滤除去三乙胺盐酸盐. 滤液浓缩后, 沉淀于乙醚两次. 过滤后的产物于室温下真空干燥至恒重, 收率为83.3%.

(ⅱ) 合成PEEP-DA. 合成聚磷酸酯均聚物: 以乙二醇为引发剂, $Sn(Oct)_2$(Sigma Aldrich)为催化剂, 在无水四氢呋喃溶液中通过EEP的开环聚合反应合成聚磷酸酯均聚物. 具体反应步骤为: 将乙二醇(124.0 mg, 2.0 mmol)、EEP(7.6 g, 0.05 mol)和$Sn(Oct)_2$(1.7 g, 4.0 mmol)溶解在无水四氢呋喃中, 于40°C反应2 h. 浓缩后, 低温下沉淀到甲苯/乙醚(4/1, v/v)混合溶剂中过滤, 室温下减压抽干至恒重, 收率为64.2%, ^{1}H-NMR检测结果表明聚合度约为22.

合成得到的聚磷酸酯均聚物两端为羟基, 进一步通过与丙烯酰氯反应, 生成聚磷酸酯二丙烯酸酯PEEP-DA. 具体反应步骤为: PEEP(3.4 g, 1 mmol)加入250 mL的三口烧瓶中, 并加入100 mL无水四氢呋喃溶液; 待完全溶解后, 将整个体系置于–5°C反应浴中, 通过恒压滴液漏斗逐滴加入三乙胺(152 mg, 1.5 mmol)和丙烯酰氯(136 mg, 1.5 mmol)的四氢呋喃溶液(20 mL). 在–5°C反应浴中, 继续反应24 h后, 过滤除去三乙胺盐酸盐. 滤液浓缩后, 沉淀于乙醚两次. 过滤后将产物于室温下真空干燥至恒重.

(ⅲ) 制备水凝胶. PEG-DA和PEEP-DA通过光交联反应制备水凝胶, 具体反应步骤为: 将不同质量比的大分子单体PEG-DA和PEEP-DA溶解于PBS中, 含量为30%(w/v), 并加入光交联剂0.05wt% I2959, 溶液置于365 nm紫外光下, 辐照强度为10.0 mW/cm^2, 时间为5 min. 根据PEEP-DA和PEG-DA单体比例, 制备得到的水凝胶分别命名为$G_{1/29}$、$G_{2/28}$、$G_{5/25}$, 表示30%(w/v)的大分子单体中, PEEP-DA和PEG-DA单体比例分别为1:29、2:28、5:25; G_{PEG}则表示大分子单体均为PEG-DA制备的水凝胶.

$G_{5/25}$复合水凝胶在PBS(0.02 mol/L, pH 7.4)、37°C条件下孵育不同时间后, 将样品冷冻干燥, 通过扫描电子显微镜(Sirion200, FEI Ltd., Hillsboro)观察其形貌. 此外, 该样品的应力-应变曲线通过Instron 3342万能材料试验机检测. 制备得到的$G_{5/25}$和G_{PEG}植入到C57BL/6小鼠背部, 28 d后取出观察. 所有小鼠均在华南理工大学实验动物中心的SPF屏障中饲养. 所有动物实验均按照华南理工大学实验动物伦理委员会批准的方案进行, 批准号2017007.

(ⅳ) 大鼠骨髓间充质干细胞(BMSCs)提取. 取6周龄的SD大鼠, 通过脱颈法处死, 在无菌条件下取大腿骨和胫骨, 无菌PBS清洗. 用剪刀剪开大腿骨和胫骨, 并用注射器吸取MSC生长培养基(含有10%FBS的α-DMEM培养基中添加20.0 μg/mL两性霉素B)冲洗, 收集骨髓, 并用移液器吹打均匀. 细胞悬浮液离心(500 g, 5.0 min), 并将沉淀重悬于新鲜的MSC生长培养基中, 并培养至T-75培养瓶中, 保持在37°C、5%CO_2和95%湿度的培养箱中. 每3天更换培养基, 当细胞达到90%左右融合时, 传代培养, 留取第4代为后续试验用.

(ⅴ) 包载BMSCs的水凝胶制备. 取第4代的鼠骨髓间充质干细胞, 重悬于大分子单体PEG-DA和PEEP-DA中(30% w/v, 光交联剂I2959含量0.05wt%), 细胞在溶液中最终浓度为1.0×10^{7}个细胞/mL; 溶液置于365 nm紫外光下, 辐照强度为10.0 mW/cm^2, 时间为5.0 min. 形成的水凝胶置于6孔板中, 并加入2.0 mL完全培养基(含有10%FBS的α-MEM培养基中添加10 mmol/L β-磷酸甘油钠、100 nmol/L地塞米松、50 mmol/L抗坏血酸).

(ⅵ) 死活染色检测. 包载BMSCs的不同水凝胶体系培养7 d后, 将水凝胶用PBS清洗2次, 并加入1.0 mL的死活染色溶液, 染色10 min后, 通过荧光显微镜(Nikon TE2000-U, 日本)检测.

(ⅶ) MTT检测. 包载BMSCs的不同水凝胶体系培养7 d后, 将水凝胶用PBS清洗2次, 并加入MTT储液(1.0 mg/mL于DMEM培养基中); 4 h后, 除去溶液, 每孔中加入600 μL萃取缓冲液(20% SDS溶解在50% *N*,*N*-二甲基甲酰胺水溶液中, pH 4.7, 37°C下配制), 通过组织匀浆机处理后, 37°C培养过夜; 离心收集上清(500 g, 10 min), 用Bio-Rad酶标仪测定570 nm处孔中溶液的吸光值. 培养1周后, 用Pierce蛋白检测试剂盒(Pierce, 美国)检测总蛋白含量.

(ⅷ) 碱性磷酸酶的活性检测. 包载BMSCs的不同水凝胶体系培养7或14 d后, PBS清洗3次, 通过组织匀浆机处理后离心收集上清(500 g, 10 min). 50 μL上清液和对硝基苯磷酸酯(Pierce)底物溶液(1.0 mg/mL, 1.0 mol/L二乙醇胺的盐酸溶液, pH 9.8)反应30 min, 使

用NaOH(50 μL, 2.0 mol/L)溶液终止反应, 用酶标仪中测其405 nm处的溶液吸光值, 以0.01 mg/mL对硝基苯酚作为参比, 总的蛋白含量通过BCA蛋白浓度测定试剂盒(Pierce)检测.

(ix) 检测钙离子含量. 包载BMSCs的不同水凝胶体系培养7或14 d后, PBS清洗3次后冻干. 随后, 不同样品中加入400 μL盐酸溶液(1.0 mol/L), 通过组织匀浆机处理, 并振荡过夜. 将最终的萃取溶液与甲基百里酚蓝(0.2 g/L)反应5 min, 用酶标仪测其612 nm波长处的溶液吸光值, 并以钙离子的标准溶液作为参比.

(x) 数据分析. 所有数据都用均数±标准差表示, 采用方差分析(ANOVA)表示是否有显著性差异; 当P<0.05时, 认为其具有显著性差异.

2 结果与讨论

2.1 大分子单体的合成与表征

聚磷酸酯通过乙二醇引发EEP的开环聚合反应得到, 其^{1}H-NMR如图1(a)所示, 链接数为22. PEG-DA和PEEP-DA分别通过PEG与PEEP的羟基与丙烯酰氯缩合反应得到, 产物^{1}H-NMR如图1(b), (c)所示. 结果显示各化学环境氢化学位移正确, 表明成功合成了PEG-DA和PEEP-DA.

2.2 水凝胶的光固化及表征

聚磷酸酯/聚乙二醇复合水凝胶制备主要在405 nm波长的激光和I2959光引发剂作用下产生自由基, 引发

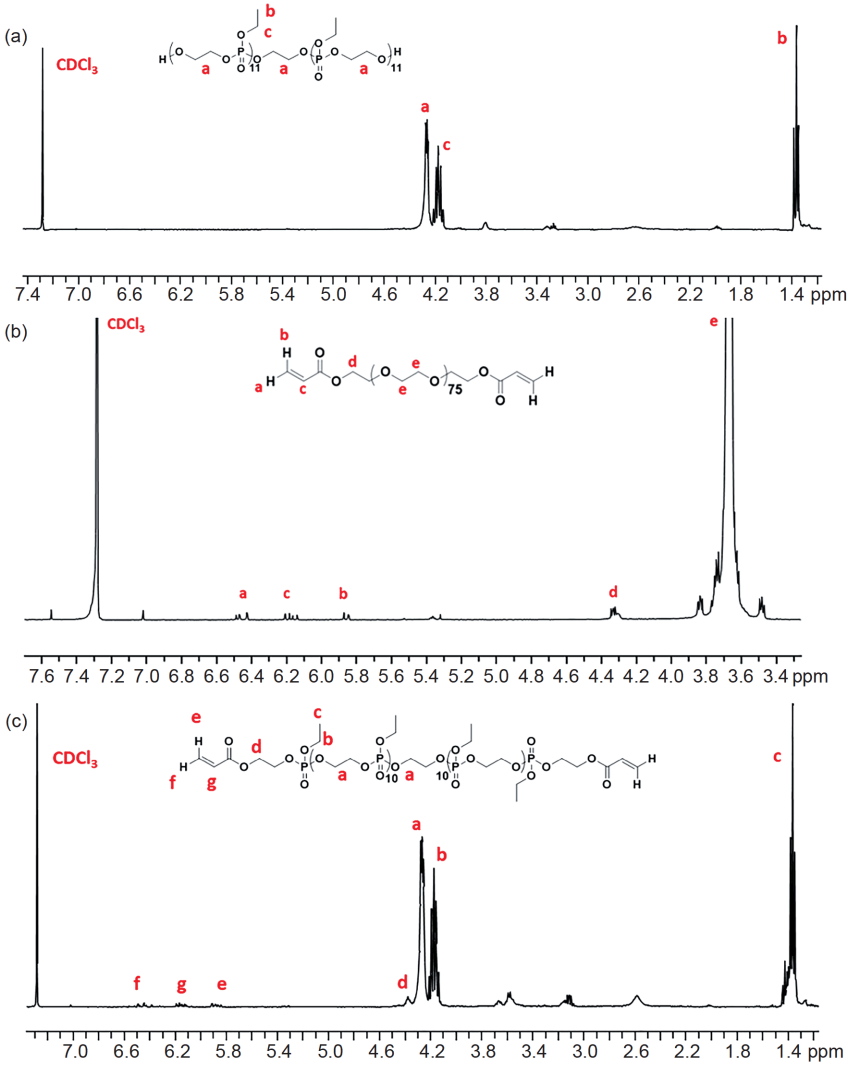

图 1 (网络版彩色)PEEP(a)、PEG-DA(b)和PEEP-DA(c)的核磁共振氢谱图
Figure 1 (Color online) 1H NMR spectrum of PEEP (a), PEG-DA (b), and PEEP-DA (c)

PEEP-DA、PEG-DA大分子单体的双键聚合. 最终形成三维网络的聚磷酸酯/聚乙二醇复合水凝胶体系. 加入不同比例的PEEP-DA后形成的水凝胶体系如图2(a)所示, 均为透明凝胶体系. 将4种不同聚磷酸酯含量的水凝胶材料置于PBS体系, 37°C下孵育28 d后, $G_{1/29}$和$G_{2/28}$复合水凝胶体系有稍许溶胀, 而$G_{5/25}$复合水凝胶最为明显, 且表面较为粗糙. 这与聚磷酸酯材料的降解性能密切相关.

为了研究$G_{5/25}$复合水凝胶的降解性能, 将该水凝胶置于PBS溶液, 并在37°C下孵育不同时间后, 检测其应力-应变曲线, 结果如图2(b)所示. 从图中可以看出, 随着孵育时间的延长, $G_{5/25}$复合水凝胶的压缩模量逐渐减小, 这是因为随着聚磷酸酯组分的逐渐降解, 水凝胶体系强度逐渐降低. 同时, $G_{5/25}$复合水凝胶在孵育不同时间后, 通过SEM表征其形貌变化. 图2(c)表明, 随着孵育时间的延长, $G_{5/25}$复合水凝胶孔隙逐渐增加, 且表面粗糙度相应增加. 此外, 将$G_{5/25}$复合水凝胶与G_{PEG}水凝胶分别植入小鼠背部, 并于28 d后观察, 可以发现$G_{5/25}$复合水凝胶基本消失(图2(d)), 而G_{PEG}水凝胶仍然保持结构的完整性, 进一步证明了$G_{5/25}$复合水凝胶的生物可降解性能.

2.3 聚磷酸酯/聚乙二醇复合水凝胶的生物相容性

在聚磷酸酯/聚乙二醇复合水凝胶光固化过程中加入BMSCs, 制备包覆细胞的水凝胶. 得到的包载BMSCs的不同复合水凝胶体系培养7 d后, 通过死活染色的方法检测其细胞活力. 结果如图3(a)所示, 聚乙二醇水凝胶G_{PEG}中, 红色的死亡细胞比例明显高于其他组; 随着复合水凝胶中聚磷酸酯材料比例的增加, 绿色的活细胞比例逐渐提高, 呈现出明显的正相关趋势.

随后, 为了进一步定量检测聚磷酸酯/聚乙二醇复合水凝胶中细胞的活力, 通过总蛋白检测方法研究培养7 d后的水凝胶中蛋白含量和活性. 结果如图3(b)所示, 培养7 d后, 复合水凝胶$G_{2/28}$和$G_{5/25}$明显优于对照的聚乙二醇水凝胶G_{PEG}, 其中$G_{5/25}$最为明显.

此外, 通过MTT法进一步研究了包覆在水凝胶中的BMSCs活力. 包载BMSCs的不同复合水凝胶体系培养7 d后加入MTT进行检测, 通过570 nm的吸光度研究不同体系中细胞的活力. 如图3(c)所示, 研究结果表明复合水凝胶$G_{2/28}$和$G_{5/25}$在570 nm的吸光度, 显著优于聚

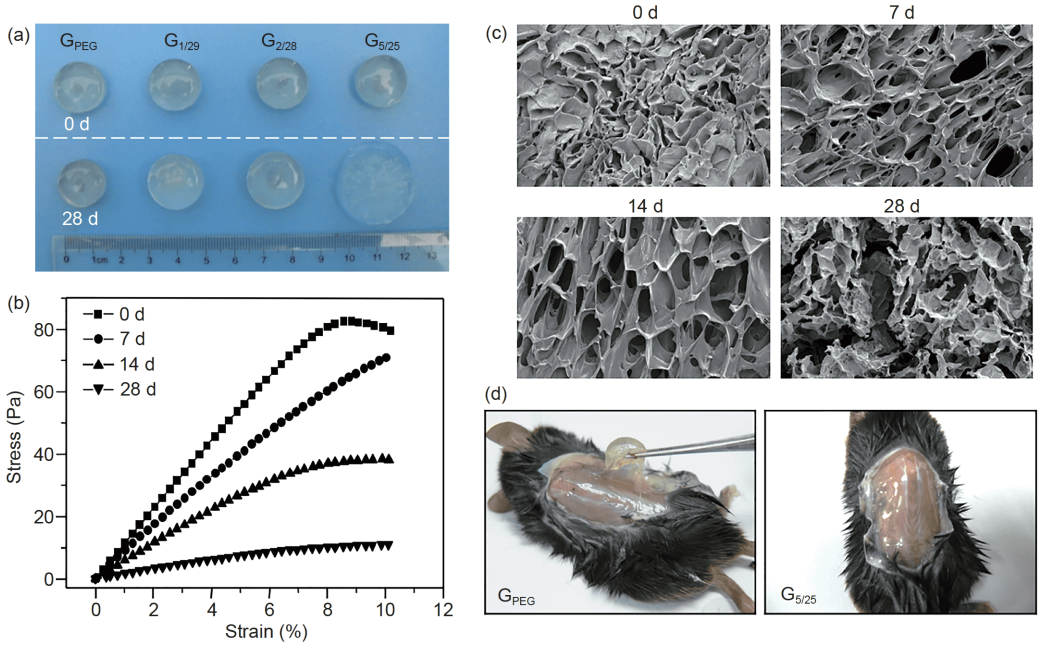

图 2 (网络版彩色)聚磷酸酯/聚乙二醇复合水凝胶制备及表征. (a) 不同比例聚磷酸酯/聚乙二醇复合水凝胶在PBS、37°C下孵育28 d后的形貌变化. $G_{5/25}$复合水凝胶PBS、37°C下孵育不同时间后的应力-应变曲线(b)和扫描电子显微镜图(SEM)(c). (d) $G_{5/25}$复合水凝胶以及G_{PEG}水凝胶体内降解

Figure 2 (Color online) Preparation and characterization of PEEP/PEG composite hydrogels. (a) Morphology changes of various PEEP/PEG composite hydrogels after 28 d of incubation at 37°C in PBS. Stress-strain curves (b) and SEM images (c) of $G_{5/25}$ hydrogels after incubation at 37°C in PBS for different time periods. (d) *In vivo* degradation of $G_{5/25}$ and G_{PEG} hydrogels

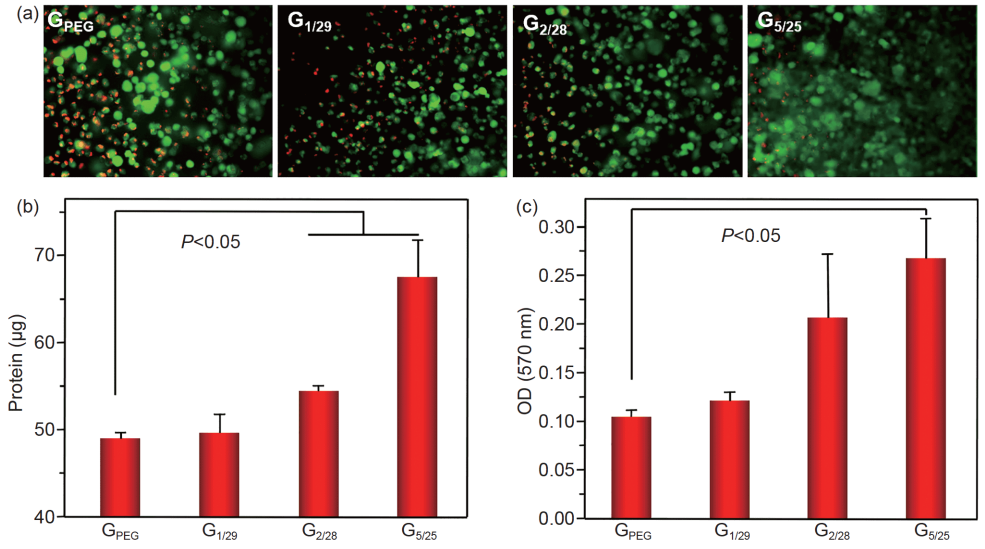

图 3 (网络版彩色)聚磷酸酯/聚乙二醇复合水凝胶的生物相容性. 包覆BMSCs的复合水凝胶培养7 d后的死活染色(a)、蛋白检测(b)和细胞活力检测(c)结果

Figure 3 (Color online) Biocompatibility of PEEP/PEG hydrogels. The live/dead stain (a), protein contents (b), and the cell viability (c) of BMSCs-encapsulating hydrogels after 7 d co-incubation

乙二醇水凝胶G_{PEG}, 进一步证明了聚磷酸酯/聚乙二醇复合水凝胶的优势.

2.4 聚磷酸酯/聚乙二醇复合水凝胶对BMSCs分化的影响

有研究表明聚磷酸酯可以增强间充质干细胞的成骨分化. 碱性磷酸酶是成骨分化的重要生物学标志物之一. 为了研究复合水凝胶对BMSCs分化的影响, 将在培养基中共培养7或14 d的复合水凝胶匀浆后, 以对硝基苯磷酸酯作为底物, 检测不同复合水凝胶体系对BMSCs分泌碱性磷酸酶的影响. 结果如图4所示, 聚磷酸酯/聚乙二醇复合水凝胶体系中, 无论是7 d还是14 d, 其碱性磷酸酶活性显著高于聚乙二醇水凝胶G_{PEG}体系, 也进一步证明了聚磷酸酯/聚乙二醇复合水凝胶在诱导BMSCs分化的优势. 但在复合水凝胶中, 聚磷酸酯的比例对碱性磷酸酶活性影响不大.

BMSCs向骨分化过程通常伴随着钙沉积增多, 而聚磷酸酯材料具有一定的骨诱导性, 有可能增强钙的沉积. 为了验证该推测, 复合水凝胶体系包裹BMSCs培养7、14和21 d后, 我们检测了不同复合水凝胶体系中的钙离子含量. 结果如图5所示, 聚磷酸酯/聚乙二醇复合水凝胶体系中, 钙沉积量显著优于聚乙二醇水凝

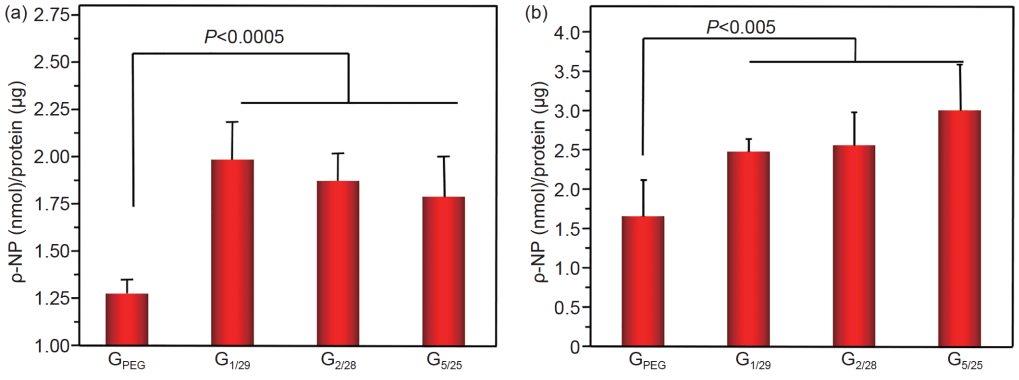

图 4 (网络版彩色)包覆BMSCs的聚磷酸酯/聚乙二醇复合水凝胶培养7(a)、14 d(b)后的碱性磷酸酶活性

Figure 4 (Color online) The alkaline phosphatase activity of BMSCs-encapsulating PEEP/PEG hydrogels after 7 (a) and 14 d (b) co-incubation

胶G_{PEG}体系, 特别是培养21 d后.

3 结论

本工作主要研究了一种聚磷酸酯/聚乙二醇复合水凝胶的制备, 及用于增强间充质干细胞的分化. 聚磷酸酯/聚乙二醇复合水凝胶通过光照下自由基聚合的方式, 引发PEEP-DA和PEG-DA聚合, 形成三维网络结构. 聚磷酸酯组分的引入, 赋予了该复合水凝胶优越的生物降解性能和生物相容性. 此外, 与聚乙二醇水凝胶相比, 聚磷酸酯/聚乙二醇复合水凝胶可以提高BMSCs分泌碱性磷酸酶, 增强钙沉积. 这些研究结果表明聚磷酸酯/聚乙二醇复合水凝胶是一种有潜力的骨再生支架材料.

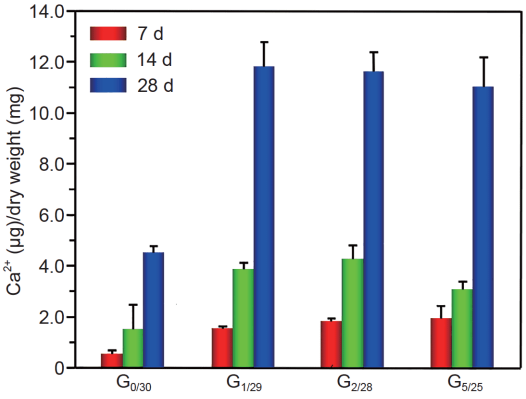

图 5 (网络版彩色)包覆BMSCs的聚磷酸酯/聚乙二醇复合水凝胶培养不同时间后的钙沉积

Figure 5 (Color online) The calcium deposition in the BMSCs-encapsulating PEEP/PEG hydrogels after co-incubation for different times

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Summary for “聚磷酸酯/聚乙二醇复合水凝胶的构建及性能”

Synthesis and characterization of polyphosphoester/polyethylene glycol composite hydrogels

Zhenghai Zhang & Xianzhu Yang*

School of Medicine, South China University of Technology, Guangzhou 510006, China
* Corresponding author, E-mail: yangxz@scut.edu.cn

The hydrogel, which has a three-dimensional (3D) cross-linked hydrophilic network structure, is widely used as biomedical scaffold for local drug delivery and tissue engineering. Especially, with the continuous development of 3D printing technology, hydrogel scaffolds are increasingly used in the field of regenerative medicine.

Polyphosphoester is a class of biodegradable polymers with repeated phosphoester linkages in the backbone, and a series of polyphosphoester materials have been synthesized for biomedical applications. For instance, a variety of amphiphilic polyphosphoester-based copolymers have been synthesized by Wang’s group, and used as the nanocarriers of hydrophobic anticancer drugs as well as siRNA. In addition, Yan’s group has prepared hyper-branched polyphosphoester as the drug delivery systems of hydrophobic anticancer drugs and photodynamic agents. Recently, Wooley and co-workers have synthesize the amphiphilic co-polymer of poly(ethylene oxide) and polyphosphoester, and used to encapsulated paclitaxel (PTX) with high contents for cancer therapy. And, they also conjugated the PTX into the side chain of polyphosphoester with pH-labile linkages for efficient tumor treatments. As the tissue engineering scaffold, polyphosphoester-based scaffolds were first used for fabrication of nerve guide conduits. And then, Elisseeff and co-workers have found the great potentials of phosphate-containing hydrogel for bone tissue engineering. In addition, Leong et al. found that the photocrosslinkable polyphosphoester hydrogel exhibited good compatibility and potentially osteoinductive ability for goat bone marrow-derived mesenchymal stem cells. Therefore, polyphosphoester hydrogel could be a promising scaffold for bone tissue engineering.

In this study, water-soluble macromers polyphosphate diacrylate (PEEP-DA) and polyethylene glycol diacrylate (PEG-DA) were first synthesized, and characterized by ^{1}H NMR. And then, the PEEP/PEG composite hydrogels at various polyphosphoester contents were controlled by varying the feed ratio of the macromers PEEP-DA and PEG-DA (1:29, 2:28, and 5:25), and the PEEP/PEG hydrogels were fabricated by exposing aqueous solutions of these macromers with photoinitiator to UV light irradiation. We systematically studied the structure, biodegradability, biocompatibility, and bone differentiation of the hydrogel scaffold. Compared with PEG hydrogel, these PEEP/PEG hydrogels exhibited better biodegradability due to the degradation properties of polyphosphoester. And, the PEEP/PEG composite hydrogels, which was prepared at PEEP-DA/PEG-DA mass ratio of 5:25, disappeared almost completely after post implantation into mice for 28 d. After encapsulating bone marrow mesenchymal stem cells (BMSCs) during the preparation process, the cell viabilities in the composite PEEP/PEG hydrogels were better than that in the control PEG hydrogel. In addition, the alkaline phosphatase activity, a key maker of early osteogenic differentiation, was evaluated after co-incubation for 7 or 14 d. It could be found that the alkaline phosphatase activity in the PEEP/PEG composite hydrogels, either for 7 or 14 d, was significantly higher than that of the control PEG hydrogel. The differentiation process of BMSCs into bone is usually accompanied by the calcium deposition. The polyphosphoester has shown osteoinductivity according to previous reports. We found that the amount of calcium deposition was significantly higher in the PEEP/PEG composite hydrogels than that in the PEG hydrogel, especially after co-incubation for 21 d, which is well consistent with the result of other groups. Collectively, this study reported a new PEEP/PEG composite hydrogels, which exhibited the potential as tissue engineering scaffolds.

polyphosphoester, hydrogel, scaffold material, biodegradable scaffold

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