doi: 10.13241/j.cnki.pmb.2017.10.004

携抗 MUC1 单克隆抗体的超声造影剂微泡制备及体外寻靶实验 *

李小宇[1] 张 鹏[1] 罗 文[2△] 谷 芬[2] 周晓东[2] 韩 璐[2] 张云飞[3]

（1 第四军医大学学员旅 陕西 西安 710032；

2 第四军医大学西京医院超声科 陕西 西安 710032；3 第四军医大学唐都医院骨科 陕西 西安 710032）

摘要 目的：制备携抗 MUCI 单克隆抗体的超声造影剂微泡，并评价其体外寻靶能力。**方法：**采用异型双功能交联剂将小鼠抗 MUCI 单克隆抗体与超声造影剂微泡相结合，采用激光粒度仪，扫描电镜及激光共聚焦显微镜评价其粒径，形态及结合率。体外培养小鼠 EMT6 细胞，将其与靶向微泡相混合，激光共聚焦显微镜评价粘附能力。**结果：**扫描电镜下显示携抗 MUCI 单克隆抗体造影剂微泡呈规则球体形态，平均粒径 2.88 ± 1.34 μm，通过异型双功能交联剂，抗 MUC1 单克隆抗体可结合至超声造影剂微泡表面，其结合率为 77.3 ± 10.4%，靶向造影剂粘附体外培养细胞比例为 79.2 ± 13.2%。**结论：**成功制备携抗 MUCI 的靶向超声造影剂微泡，并且能很好的识别粘附体外培养乳腺癌细胞。

关键词：MUC1；超声造影剂；乳腺癌

中图分类号：R-33; R445.1; R737.9 **文献标识码：**A **文章编号：**1673-6273(2017)10-1815-03

Preparation of Ultrasound Contrast Agent Microbubbles Combining Anti-MUC1 Monoclone Antibody and Evaluation of Targeting Capability in Vitro*

LI Xiao-yu[1], ZHANG Peng[1], LUO Wen[2△], GU Fen[2], ZHOU Xiao-dong[2], HAN Lu[2], ZHANG Yun-fei[3]

(1 Student Bragade, Fourth Military Medical University;

2 Department of Ultrasound, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, 710032, China;

3 Department of Orthopaedics, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi, 710032, China)

ABSTRACT Objective: To prepare ultrasound contrast agent microbubbles combining anti-MUC1 monoclone antibody and evaluate its targeting capability in vitro. **Methods:** Ultrasound contrast agent microbubbles and anti-MUC1 monoclone antibodies were combined via N-succinimide 3- (2-pyridyl dithio) propionate (SPDP). Laser particle size analyzer, scanning electron microscope (SEM) and laser scanning confocal microscope (LSCM) were used to assess the diameter, morphology and ratio of combination between microbubbles and antibodies. Mice EMT6 breast cancer cells were cultured in vitro, which were then mixed with MUC1-targeted microbubbles. Adhension rate were evaluated via LSCM. **Results:** SEM showed MUC1-antibody carrying microbubbles were regular spherical particles with the diameter of 2.88 ± 1.34 μm. Via SPDP, MUC1-antibodies were conjugated onto the surface of microbubbles with the ratio of 77.3 ± 10.4% by LSCM. Adhension rate was 79.2 ± 13.2%, when MUC1-antibody carrying microbubbles were mixed with Mice EMT6 breast cancer cells cultured in vitro. **Conclusions:** MUC1-antibody carrying microbubbles were prepared successfully, and could adhere to breast cancer cells in vitro.

Key words: MUC1; Contrast agent microbubbles; Breast cancer

Chinese Library Classification(CLC): R-33; R445.1; R737.9 Document code: A

Article ID: 1673-6273(2017)10-1815-03

前言

乳腺癌是危及女性健康的常见肿瘤之一，其发病率在欧美发达国家多居女性肿瘤的首位。该病近年来在全球有明显上升趋势，也正迅速成为中国妇女主要死亡原因之一[1,2]。研究发现携带肿瘤细胞或新生血管标志物特异性抗体的超声造影剂微泡，能够对肿瘤细胞或新生血管特异性识别，在提高肿瘤组织的靶向显影，增强影像学手段对良恶性判断以及介导药物基因治疗等方面具有重大意义[3,4]。MUC1 是一种高表达于乳腺癌上皮组织中的高分子量蛋白，其跨膜序列如棒状插入细胞膜，并有一个由 69 个氨基酸残基组成的尾延伸到细胞质中[5,6]。MUC1 在乳腺癌中高度异常表达． 由于其特异性高于组织多肽抗原．敏感性高于癌胚抗原(CEA)，所以对于乳腺癌的诊断具有潜在的临床应用价值[7]。本研究拟采用异型双功能交联剂制备携载抗 MUC1 单克隆抗体的靶向超声造影剂微泡，并评价其体外寻靶能力。

* 基金项目：国家自然科学基金项目(81000918)；陕西省自然科学基金项目(2014JQ4166)

作者简介：李小宇(1995-)，本科，研究方向：超声介入及分子影像，电话：029-84775447，E-mail: 358830385@qq.com

△ 通讯作者：罗文，主治医师，讲师，主要研究方向：超声介入及分子影像，E-mail: 503883752@qq.com

（收稿日期：2016-10-19 接受日期：2016-11-13）

1 材料与方法

1.1 材料

1.1.1 主要试剂与仪器 小鼠 MUC1 单克隆抗体购自 Antibody Diagnostica 公司。超声造影剂微泡(Bracco, Italy),异型双功能交联剂 (Merk, Germany)。胰蛋白酶,RPMI1640 培养基,10%胎牛血清(GIBCO)。激光粒径测量仪(Malvern,UK),扫描电镜(HitachiS4800, Japan),激光共聚焦显微镜(Nikon,Japan)。

1.1.2 细胞系小鼠乳腺癌细胞系 EMT6 为第四军医大学实验动物中心提供。

1.2 方法

1.2.1 细胞培养 EMT6 细胞经胰蛋白酶消化传代后,接种 RPMI1640 培养基中,培养基加 10%胎牛血清、50 μg/m L 青霉素和 100 μg/m L 链霉素,在含 5%CO_2 细胞培养箱内 37 ℃ 培养:倒置显微镜观察细胞生长状态。实验中所用细胞均取对数生长期 EMT6 细胞。

1.2.2 超声造影剂 Sonovue 配置 将 5 mL 0.9% 生理盐水加入至超声造影剂 Sonovue 冻干粉瓶中,轻轻摇匀,即配置成 Sonovue 混悬液。

1.2.3 携 MUC1 单抗的靶向造影剂制备 取适量的小鼠 MUC1 单克隆抗体(0.2 mL 浓缩液, 200 倍稀释),以 1:15 的比例加入 20 mmol/LSDPD 溶液,置于 4℃反应 1h,再离心洗涤 5 次(6000 r/min)。加入二硫苏糖醇醋酸溶液,置于 4℃反应 30 min,再离心洗涤三次,得到带巯基的抗体溶液。超声造影剂微泡(SonoVue, Bracco)混悬液与适量 20 mmol / LSDPD 溶液混合,置于 4℃反应 1 h,然后用磷酸盐缓冲液洗涤,得到吡啶二硫基的微气泡悬液,然后与抗体溶液相混合,置于 4℃反应过夜,再用磷酸盐缓冲液洗涤,最终得到携载 MUC1 抗体的靶向超声造影剂微泡。

1.2.4 携 MUC1 单克隆抗体的靶向微泡粒径检测及形态观察 激光粒度仪检测靶向微泡粒径大小。将样品滴在有支持膜的铜网上,用磷钨酸负染,观察靶向微泡的形态结构。FITC 标记 MUC1 单抗,罗丹明标记超声靶向微泡,激光共聚焦显微镜下观察,并计算抗体与微泡的结合率:结合抗体的微泡个数 / 未结合抗体的微泡个数× 100%。共 10 批样品,每批样品随机观察 10 个高倍视野(× 400),然后取平均值。

1.2.5 体外寻靶实验 将罗丹明标记的靶向微泡与体外培养小鼠 EMT6 乳腺癌细胞(吖啶橙染色)进行混合,激光共聚焦显微镜观察微泡粘附比率:粘附细胞的靶向微泡个数 / 未粘附细胞的靶向微泡个数× 100%。共 10 批样品,每批样品随机观察 10 高倍视野(× 100),然后取平均值。

1.3 统计分析

采用 SPSS 13.0 分析软件。所有计量数据均计算平均值及标准差,采用 ($\bar{x}\pm s$) 表示,样品多组间比较采用单因素方差分析,P<0.05 被认为有统计学差异。

2 结果

2.1 携抗 MUC1 抗体的超声造影剂微泡形态学及粒径检测

扫描电镜下观察携抗 MUC1 抗体的超声造影剂微泡可维持球体形态,并且形态规则(图 1)。通过对 10 个批次的样本进行检测,激光粒度仪测量散在粒径分布范围约为 1 -12 μm,平均粒径 2.88 ± 1.34 μm (图 2),65%-80%的微泡粒径小于 5 μm,各批次样本间无明显统计学差异(P>0.05)。由此可见本研究制备的携抗 MUC2 抗体的超声造影剂微泡形态稳定,粒径均匀。

2.2 携抗 MUC1 抗体的超声造影剂微泡抗体结合率及体外寻靶能力

激光共聚焦显微镜下,绿色的 MUC1 单克隆抗体附着于红色的超声造影剂微泡表面。超声造影剂微泡与 MUC1 单克隆抗体结合率为 77.3 ± 10.4%(图 3),各批次样本间无明显统计学差异(P>0.05)。而体外寻靶实验中,取对数期生长的小鼠 EMT6 乳腺癌细胞,并使其处于悬浮状态。激光共聚焦显微镜显示携抗 MUC1 单克隆抗体的靶向造影剂微泡呈红色,粘附于呈黄绿色的小鼠 EMT6 乳腺癌细胞(图 4),粘附结合率达到 79.2 ± 13.2%。由此可见本研究制备的携抗 MUC2 抗体的超声造影剂微泡具备较好的体外寻靶能力。

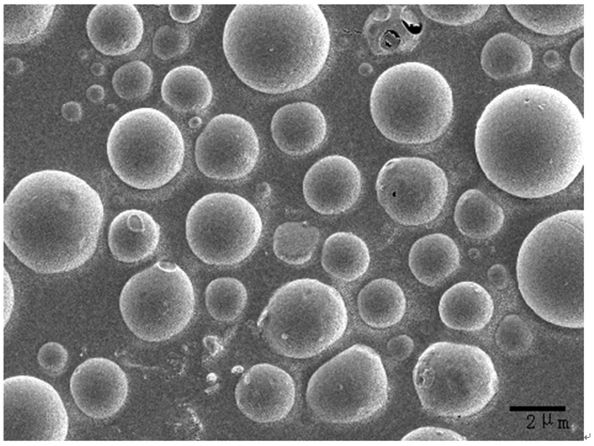

图 1 扫描电镜示携抗 MUC1- 超声造影剂微泡呈规则球形

Fig. 1 MUC1 antibody carrying microbubbles presented as regular sphere shape on SEM

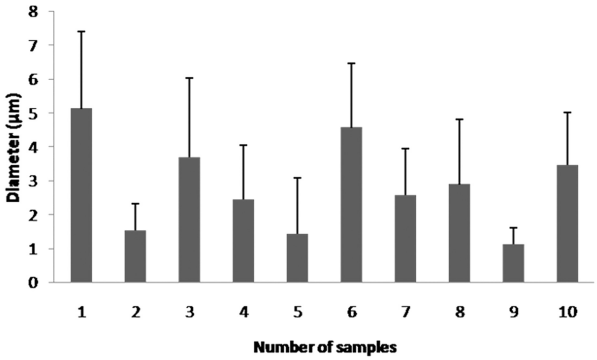

图 2 10 批携抗 MUC1- 超声造影剂微泡样品粒径分布

Fig.2 Diameter of MUC1 antibody carrying microbubbles for ten samples

3 讨论

超声造影剂微泡内部包含气体,外部具有壳膜结构,直径小于红细胞直径,能够顺利通过毛细血管网等微循环结构[8]。超声造影剂微泡能够通过增强红细胞的背向散射,从而增加超声

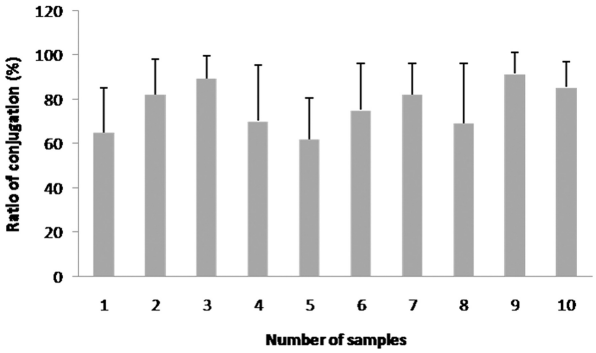

图 310 批携抗 MUC1- 超声造影剂微泡样品中抗体与微泡的结合率

Fig 3Ratio of MUCI antibody conjugated to microbubbles for ten samples

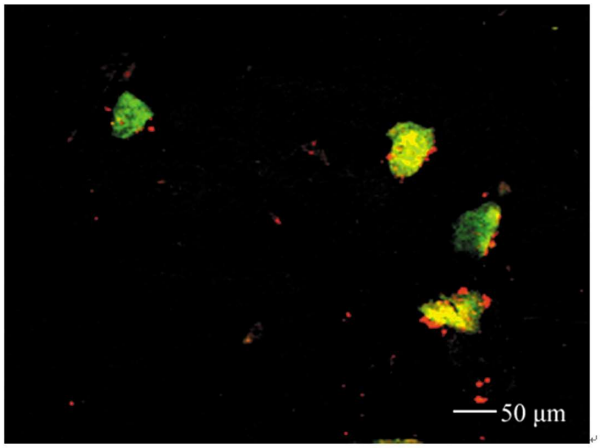

图 4 激光共聚焦显微镜示体外培养小鼠 EMT6 乳腺癌细胞(绿色)与携抗 MUC1- 超声造影剂微泡(红色)相结合

Fig.4 Ratio of MUC1 antibody carrying microbubbles(red) adhering to mice EMT6 breast cancer cells (green) in vitro

回声信号,从而显示血流分布及血流动力学变化[9,10]。因为具有无辐射、无创、可重复性高及过敏性低等优势,目前超声造影成像已广泛的应用于临床肝、肾、甲状腺等脏器肿瘤的良恶性诊断中,并成为鉴别诊断的重要方法[11-13]。

近年来,靶向微泡的研究备受关注。超声造影剂微泡通过携带特异的抗体从而增强肿瘤组织区域的特异性显影,用以观察靶组织在细胞及亚细胞水平的成像,反映病变组织在分子基础上的变化,并且能够提供靶向释放药物的途径,成为分子影像领域的研究热点[14-16]。国内外学者尝试了多种方法制备靶向超声造影剂微泡。在较早的时候,Ellegala 等将一种特异性与整合素 alpha(V)beta(3)绑定的抗体,重组蛇毒锯鳞蝰素(Echistatin),与微泡相连结,制得抗 alpha(V)beta(3)靶向微泡,用以显像大鼠恶性神经胶质瘤模型,结果显示胶质瘤明显强化[17]。另外,有人采用共价结合法制备人卵巢癌细胞靶向超声造影剂即黄体生成素释放激素一脂质体微泡(LHRH-LM),倒置显微镜下观察到靶向微泡与人卵巢癌细胞株 OVCAR-3 细胞能高效的粘附结合[18]。

目前针对乳腺癌的靶向微泡尚报道不多,靶点多选用肿瘤新生血管相关的特异性标志物,包括 VEGFR2 等[19],尚未见直接针对乳腺癌细胞的标志物报道。而本研究直接采用针对乳腺癌细胞的标志物,在特异性上将会有很大提高。另外在抗体和微泡结合方面,国内外学者多采用生物素法,而这一方法需要新鲜制备微泡,而不能采用成品微泡。异型双功能交联剂的使用,可以使抗体直接连接于成品微泡表面,使得靶向微泡制备进一步简化。近来,国内学者报道了采用异型双功能交联剂成功制备了 IL-8 抗体偶联的超声造影剂微泡,加入二抗后其凝集反应为阳性[20,21]。

在本研究中,针对乳腺癌上皮组织高度表达的 MUC1 蛋白,我们采用异型双功能交联剂将小鼠抗 MUC1 抗体与超声造影剂微泡相偶联,制备成靶向造影剂,靶向造影剂在体外寻靶实验中,能够较好的和小鼠 EMT6 乳腺癌细胞粘附,为乳腺癌的超声分子影像精准诊断及靶向释药提供新的途径。扫描电镜结果证实偶联特异性抗体后的超声造影剂微泡仍然能保持较规则的球体形态,并且维持较小的粒径,这些特征为进一步体内超声显影提供了基础。本研究中所制备的携抗 MUC1 抗体的靶向造影剂虽然在体外寻靶实验中能够与乳腺癌细胞很好的粘附。本研究中携抗 MUC1 抗体的靶向造影剂靶向于乳腺癌细胞,在局部注射的情况下,可与乳腺癌细胞相结合,但是如果通过血液循环进入体内,则它自身无法通过血管内皮屏障,进入组织间隙,这可能首先要通过超声空化等方法开放血管内皮屏障,然后帮助靶向微泡进入组织内寻找靶细胞。携抗 MUC1 抗体的靶向微泡在体内环境中的寻靶功能及显影效果尚需要进一步进行动物实验验证。

综上所述,本研究成功制备了携抗 MUCI 的靶向超声造影剂微泡,并且能够在体外很好的识别培养乳腺癌细胞。

参 考 文 献 (References)

[1] Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008[J]. CA Cancer J Clin, 2008, 58(2): 71-96

[2] Di Sibio A, Abriata G, Forman D, et al. Female breast cancer in central and south america [J]. Cancer Epidemiol, 2016, 44 (Suppl 1): S110-S120

[3] Hauff P, Reinhardt M, Foster S. Ultrasound contrast agents for molecular imaging[J]. Handb Exp Pharmacol, 2008,(185 Pt 1): 223-245

[4] Rychak JJ, Graba J, Cheung AM, et al. Microultrasound molecular imaging of vascular endothelial growth factor receptor 2 in a mouse model of tumor angiogenesis[J]. Mol Imaging, 2007, 6(5): 289-296

[5] Xia J, Shi J, Wang P, et al. Tumour-associated autoantibodies as diagnostic biomarkers for breast cancer: A systematic review and meta-analysis[J]. Scand J Immunol, 2016, 83(6): 393-408

[6] Piña MJ, Girotti A, Santos M, et al. Biocompatible ELR-based polyplexes coated with MUC1 specific aptamers and targeted for breast cancer gene therapy[J]. Mol Pharm, 2016, 13(3): 795-808

[7] Laidi F, Bouziane A, ErrachidA, et al. Usefulness of salivary and serum auto-antibodies against tumor biomarkers HER2 and MUC1 in breast cancer screening [J]. Asian Pac J Cancer Prev, 2016, 17(1): 335-339

[8] Caschera L, Lazzara A, PiergalliniL. Contrast agents in diagnostic imaging: Present and future[J]. Pharmacol Res, 2016, 110: 65-75

[9] Nolsøe CP, LorentzenT. International guidelines for contrast-enhanced ultrasonography: ultrasound imaging in the new millennium [J]. Ultrasonography, 2016, 35(2): 89-103

(下转第 1917 页)

predict deep deltoid ligament incompetence in supination-extemal rotationtype ankle fractures [J]. J Orthop Trauma, 2013, 21 (4): 244-247

[8] von Recum J, Wendl K, Vock B, et al. Intraoperative 3D C-arm imaging. State of the art[J]. Unfallchirurg, 2012, 115(9): 196-201

[9] Michelson JD, VamerKE, Checcone M. Diagnosing deltoid injury in ankle fractures: the gravity stress view [J]. Clin Orthop Relat Res, 2011, 387: 178-182

[10] Henari S, Banks LN, Radovanovic I, et al. Ultrasonography as a diagnostic tool in assessing deltoid ligament injury in supination external rotation fractures of the anki [J]. Orthopedics, 2011, 34(10): 639-643

[11] Deland JT, de Asia RJ, Segal A. Reconstruction of the chronically failed deltoid ligament:a new technique [J]. Foot Ankle Int, 2014, 25 (11): 795-799

[12] Boss AP, Hintermann B. Anatomical study of the medial ankle ligament complex[J]. Foot AnkleInt, 2012, 23(6): 547-553

[13] Beerekamp MS, Sulkers GS, Ubbink DT, et al. Accuracy and consequences of 3D-fluoroscopy in upper and lower extremity fracture treatment: a systematic review [J]. Eur J Radiol, 2012, 81 (420): 4019-4028

[14] Schuberth JM, Collman DR, Rush SM, et al. Deltoid ligament integrity in lateral malleolar fractures: a comparative analysis of arthroscopic and radiographic assessments[J]. Foot Ankle Surg, 2014, 43(1): 20-29

[15] Ruan Z, Luo C, Shi Z, et al. Intraoperative reduction of distal tibiofibular joint aided by three-dimensional fluoroscopy [J]. Technol Health Care, 2011, 19(3): 161-166

[16] Giovinco NA, Dunn SP, Dowling L, et al. A novel combination of printed 3-dimensional anatomic templates and computer-assisted surgical simulation for virtual preoperative planning in Charcot foot reconstruction[J]. Foot Ankle Surg, 2012, 5-6(3): 387-393

[17] Dubois-Ferriè re V, Assal M. Benefit of computer assisted surgery in foot and ankle surgery[J]. Rev Med Suisse, 2014, 10(420): 562-564

[18] Brunner A, Heeren N, Albrecht F, et al. Effect of three-dimensional computed tomography reconstructions on reliability [J]. Foot Ankle Int, 2012, 33(9): 727-733

[19] Gay SB, Sistrom C, Wang GJ, et al. Percutaneous srew fixation of acetabular fracture with CT guidancerrpreliminary results of a new teachnique[J]. AJR Am J Roentgenol, 2012, 158: 819

[20] 魏国柱, 高红, 刘曦, 等. 螺旋 CT 扫描及三维重建技术在股骨颈骨折分型及治疗中的应用 [J]. 现代生物医学进展, 2016, 16(10): 1946-1948

Wei Guo-zhu, Gao Hong, Liu Tun, et al. Application of Spiral CT Scanning and 3D Reconstruction Technology in Classification and Treatment of Femoral Neck Fracture [J]. Progress in Modern Biomedicine, 2016, 16(10): 1946-1948

(上接第 1817 页)

[10] Paefgen V, Doleschel D, KiesslingF. Evolution of contrast agents for ultrasound imaging and ultrasound-mediated drug delivery [J]. Front Pharmacol, 2015, 15: 197

[11] Maruyama H, Sekimoto T, Yokosuka O. Role of contrast-enhanced ultrasonography with Sonazoid for hepatocellular carcinoma: evidence from a 10-year experience [J]. J Gastroenterol, 2016, 51(5): 421-433

[12] Dong Y, Wang WP, Lin P, et al.Assessment of renal perfusion with? contrast-enhanced ultrasound: Preliminary results in early diabetic nephropathies [J].ClinHemorheolMicrocirc, 2016, 62(3): 229-238

[13] Maruyama H, Sekimoto T, Yokosuka O. Role of contrast-enhanced ultrasonography with Sonazoid for hepatocellular carcinoma: evidence from a 10-year experience [J]. J Gastroenterol, 2016, 51(5): 421-433

[14] Xu J, Zeng X, Liu Y, et al.A novel dual-targeted ultrasound contrast agent provides improvement of gene delivery efficiency in vitro [J]. TumourBiol, 2016, 37(7): 8609-8619

[15] Castle J, Feinstein SB. Drug and gene delivery using sonoporation for cardiovascular disease[J]. AdvExp Med Biol, 2016, 880: 331-338

[16] Abou-Elkacem L, Bachawal SV, WillmannJK. Ultrasound molecular imaging: Moving toward clinical translation [J]. Eur J Radiol, 2015, 84(9): 1685-1693

[17] Ellegala DB, Leong-Poi H, Carpenter JE, et al. Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to alpha(V)beta(3)[J]. Circulation, 2003, 108(3): 336-341

[18] 贺娟,孙江川,常淑芳, 等. 人卵巢癌靶向超声造影剂的制备及其体外寻靶能力观察[J]. 中国医学影像技术, 2009, 25(6): 929-931

He Juan, Sun Jiang-chuan, Chang Shu-fang, et al. Preparation of targeted liposome microbubbles and observation of its targeted ability in human ovarian cancer in vivo [J]. Chinese Journal of Medical Imaging Technology, 2009, 25(6): 929-931

[19] Abou-Elkacem L, Wilson KE, Johnson SM, et al. Ultrasoundmolecular imaging of the breast cancer neovasculature using engineered fibronectin scaffold ligands: A novel class of targeted contrast ultrasound agent [J]. Theranostics, 2016, 6 (11): 1740-1752

[20] 杨薇,陆永萍,邵庆华, 等. IL-8 单克隆抗体靶向偶联 Sonovue 微气泡造影剂的方法学研究 [J]. 中国超声医学杂志, 2011, 27(6): 485-488

Yang wei, Lu Yong-ping, Shao Qing-hua, et al. Methodology of anti-human interleukin-8 monoclonal antibody targeted conjugating SonoVuemicrobubbles contrast agent [J]. Chinese Journal of Ultrasound in Medicine, 2011, 27(6): 485-488

[21] 邵庆华,李云燕,杨薇, 等.SonoVue 与 IL-8 单克隆抗体偶联对兔腹主动脉粥样斑块造影的研究[J].华中科技大学学报(医学版), 2013, 42(5): 573-577

Shao Qing-hua, Li Yun-yan, Yang Wei, et al. Targeted imaging of atherosclerotic plaque in the rabbit abdominal aorta with SonoVue conjugated with IL-8 monoclonal antibody [J]. Journal of Huazhong University of Science and Technology: Medical Sciences, 2013, 42 (5): 573-577