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Radiolabeling of Filamentous Phage Peptide Library With ^{99m}Tc and Its Biodistribution in Normal Mice*

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Abstract Numerous peptides that bind to a given target have been selected by phage display technology. However, some peptides isolated to date do not bind with high affinity to tumor or organ sites, even peptides were selected *in vivo*. Therefore, the biodistribution of ^{99m}Tc-labeled filamentous phage peptide library via MAG3 (mercaptoacetyltriglycine) were investigated to gain a better understanding of phage circulation *in vivo*. The experimental results showed that the liver and spleen were the organs of the greatest accumulation, while heart, muscle, pancreas and brain retained less radioactivity. In opposite to other tissues and organs, the radioactivity in stomach, intestine and bone gradually went up with time. The clearance of ^{99m}Tc-labeled phage in blood was very fast from 5 min to 30 min and then slowed down. When phage *in vivo* circulated at enough long period of time, some phage particles could extravasate in some organs or tissues and internalized there. In conclusion, the circulation time of phage *in vivo* should be experimentally determined beforehand according to the targeted organs and the specific location of target peptides in order to panning a peptide with high specificity and affinity to that target.

Key words filamentous phage display library, biodistribution, labeling, ^{99m}Tc

Since Smith[1] originally developed phage vector system in 1985, enormous efforts have been made in the application of this method in selection of tissue- or tumor-targeting agents[2~10]. Many peptide ligands had been acquired. The RGD, an excellent tumor-targeting peptide was obtained in this way[11, 12]. However, although many peptides often possess high affinity to their targeted receptors *in vitro*, they may not effectively target desired tissues *in vivo*, even though these peptides were identified *in vivo*[13]. To find out why there are such issues, researchers have done much work by using longer panning time, by displaying peptides with different number of amino residues with or without conformational constraint, even by complementarily using polyvalent and monovalent display libraries or landscape libraries[14]. Deutscher *et al*[15, 16]. thought that phage selection in animals had been performed for too short time without optimization for biodistribution or clearance rates to a particular organ.

We also met the similar problem when we carried out screening tumor- or organ-specific peptides with filamentous phage peptide library. Therefore, we turn to check the biodistribution of filamentous phage peptide library itself so as to get better understanding for phage circulating *in vivo*. There have been some reports about the biodistribution *in vivo* of different phage types. Inchley reported that ^{51}Cr-T4 phage was immediately cleared to the liver[17]. Geier *et al*[18] found there was the highest titer for lambda phage in spleen and was cleared much more slowly than in other organs. Ruschowski's[19] study showed that ^{99m}Tc-labeled bacteriophage is a potential infection-specific imaging agent. Yip *et al*[20] studied the biodistribution of filamentous phage-Fab in nude mice. The study indicated that predominant phage titer occurred in lung, kidney, liver and spleen. The serum half-life of the phage was found to be ~3.6 h.

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Immunohistochemical analysis showed phage to be mainly within vasculature at 4 min, whereas notable phage extravasation was observed at 24 h and 72 h. Zou *et al* [16] first studied the biodistribution of filamentous phage peptide library in different types of mice by detecting phage titer in 2004. The article reported that the serum half-life of engineered phage was found to be ～12 min, 16 min and 18 min respectively in normal mice, in SCID (severe combined immune deficiency) and in nude mice. Predominant phage uptake still occurred in lung, kidney, and liver as well as spleen. Molenaar's report [21] showed the low recovery of CFUs (colony forming units) as compared to the radioactivity of ^{35}S-labeled phage as bacteriophage M13 was rapidly internalized and degraded *in vivo* within 30～90 min after injection and the recovery of infectious phage decreased dramatically after 10 min or 30 min (time was different as organs). That CFU values will provide considerable underestimation of real uptake stresses the importance of radiolabeling for quantitative assessment of phage. It is essential to measure total amount of tissue-associated phage particles by activity rather than by colony forming units. The application of ^{35}S-labled phage has some limitations because of the longer labeling time (labeling cultures grown overnight at 37℃), the more complicated labeling process (metabolically labeled phage with ^{35}S) and ^{35}S-labled phage with the lower specific activity.

In this article, the biodisribution of ^{99m}Tc-labeled phage via MAG3 was studied due to ^{99m}Tc-labeled phage with higher specific activity, simpler labeling process and shorter labeling time.

1 Materials and methods

1.1 Materials and equipments

All reagents were of A. R. grade and were used without further purification. All water used was de-ionized. The Ph.D-12 phage display peptide library kit (containing random 12 peptides) was purchased from New England Biolabs (NEB). LB medium (10 g Bacto-Tryptone, 5 g yeast extract and 5 g NaCl in 1 L distilled water), tetracycline stock, PEG/NaCl, TBS (Tris-HCl，0.15 mol/L NaCl, pH 7.5) and PBS (phosphate-buffered saline, pH 7.4) were prepared according to procedures recommended in the NEB product manual. Bacto-tryptone, yeast extract, NaCl, DFM (N, N-dimethylformamide), PEG (polyethylene glycol with average M = 8 000) and agar were purchased from Beijing Xin Jing Ke Biotechnology Co. Ltd (Beijing, China). DCC (N, N- dicyclohexylcarbodiimide), NHS (N-hydroxylsuccinimide) was from Sigma Chemical Co. MAG3 was synthesized by our library.

The ^{99m}Tc-pertechnetate was eluted from a ^{99}Mo-^{99m}Tc generator (China Institute of Atom Energy, Beijing). Radioactivity in the organs of mice was assayed by a Cobra Ⅱ series Auto-Gamma Counting System (Ramsey, USA). The radioactivity of ^{99m}Tc-labeled products was determined by a FT-603 γ well counter (Beijing Nuclear Instrument Factory, China). High-Speed Refrigerated Centrifuge was produced by HITACHI (Japan). Isothermal Vibrator was made by Taichang Experiment Instrument Factory (Jiangsu, China). The solution of sodium tartrate used in this study was made up of sodium tartrate (50 g/L) in 0.5 mol/L sodium bicarbonate, 0.25 mol/L ammonium acetate and 0.175 mol/L ammonium hydroxide buffer solution (pH 9.2).

Kunming mice (18～20 g, male) were supplied by the Breeding Center of Zoology from Peking University Health Science Center. All experiments were carried out following the principles of laboratory animal care and regulations for the administration of affairs concerning experimental animals (China).

1.2 Experimental methods

1.2.1 Phage amplification. Ph.D-12 phage display peptide library (100 μl) was incubated with 1 ml of fresh *E. coli* ER2738 for 0.5 h. LB-Tet medium (0.2 μg tetracycline/ml LB, 10 ml) was added to the mixture. Then, the mixture was incubated at 37℃ with vigorously shaking for 4.5 h. The culture was spun for 10 min at 10 000 r/min at 4℃. The supernatant was transferred to a fresh tube and re-spun. The upper 80% of the supernatant was moved to a fresh tube and 1/6 volume of PEG/NaCl was added. The phage was allowed to precipitate at 4℃ for 1 h. The PEG precipitation was spun for 15～20 min at 11 000 r/min at 4℃. The supernatant was discarded and the residual suspension was re-spun briefly, then the residual supernatant was removed with pipette. The pellet was suspended in 100 μl of TBS and purified once again with PEG/NaCl. The amplified phage was suspended in PBS and stored at 4℃.

1.2.2 Synthesis of S-acetyl NHS-MAG3. Synthesis procedures were done according to reference[22] with some modifications. S-acetyl MAG3 (99.2 mg) and NHS (39 mg) were dissolved in 3 ml of DMF. The

reaction mixture was placed in an ice bath, 1 ml of DMF containing DCC (70.7 mg) added. DMF (200 μl) containing DCC (8 mg) was again added to the mixture after stirring for 1 h and the mixture was continuously stirred for 20 h. The precipitation was filtered and ether (60 ml) was added to the filtrate and stirred for about 40 min. The precipitation was filtered. The residue was again dissolved in 2 ml of DMF and was crystallized from ether. The yield was 83.4%.

1.2.3 Conjugation of phage with MAG3. Conjugation procedures were done according to reference[19] with some modifications. The phage was conjugated with S-acetyl NHS-MAG3. To 100 μl of PBS containing about 10^{11} pfu/ml of phage was added 3 μl of 0.1 mol/L aqueous $NaHCO_3$ solution (pH 9.0) to make the final pH 8.0. With continuous agitation, 4 μl of fresh solution of NHS-MAG3 in dry DMF (1 g/L) were added. One-sixth volume of PEG/NaCl solution was added to the mixture after it was incubated at room temperature for 45 min, and then incubated on ice for more than 15 min. The PEG precipitation was spun for 15～20 min at 11 000 r/min at 4℃. MAG3-phage pellet was suspended in PBS and precipitated once again with PEG/NaCl. Final pellet was suspended in PBS and stored at 4℃.

1.2.4 Radiolabeling of MAG3-phage with ^{99m}Tc. Radiolabeling procedures were done according to reference[19] with some modifications. To 40 μl of MAG3-phage (10^{10} pfu/ml) was added 3.7×10^7 Bq [^{99m}Tc]- pertechnetate and 12 μl of aqueous sodium tartrate solution and 8 μl of fresh stannous chloride solution (2 mg $SnCl_2\cdot2H_2O$/ml in 10 mmol/L HCl). The mixture was incubated at room temperature for 45 min. The ^{99m}Tc-labeled MAG3-phage was purified by precipitation twice with PEG/NaCl as described above. TLC system was used to identify the labeled products (fixed phase/developer): chromatographic paper (Xinhua No. 1)/saline.

1.2.5 Biodistribution. Kunming mice were randomly allocated into 4 groups, five for each group. Aliquots (100 μl, about 10^9 pfu/ml) of 962 kBq ^{99m}Tc-MAG3-phage, was injected via tail vein without anesthesia for each mouse. Mice injected ^{99m}Tc-MAG3-phage was sacrificed by dislocation cervical at 5 min, 30 min, 1 h and 2 h post injection. The organs or tissues of interest were removed, washed, and weighed prior to radioactivity counting. The same injection solution was taken as a standard for calculating the percent-injected dose per gram of tissue, i.e., % ID/g tissue. The final results were expressed as $\bar{x} \pm s$. Tissues or organs: blood ratios of ^{99m}Tc-MAG3-phage at various points (abbreviated as organs or tissues/blood afterwards for simplification) were calculated by dividing the radioactivity concentration in respective organ (in % ID/g) at specified time points by that in blood at same time points.

2 Results

2.1 Radiochemical purity of ^{99m}Tc-labeled MAG3-phage

After the ^{99m}Tc-labeled MAG3-phage was purified by precipitation with PEG/NaCl and centrifugation twice, all other components should be remained in supernatant except ^{99m}Tc-labeled MAG3-phage. The radiochemical purity of ^{99m}Tc-MAG3-phage was higher

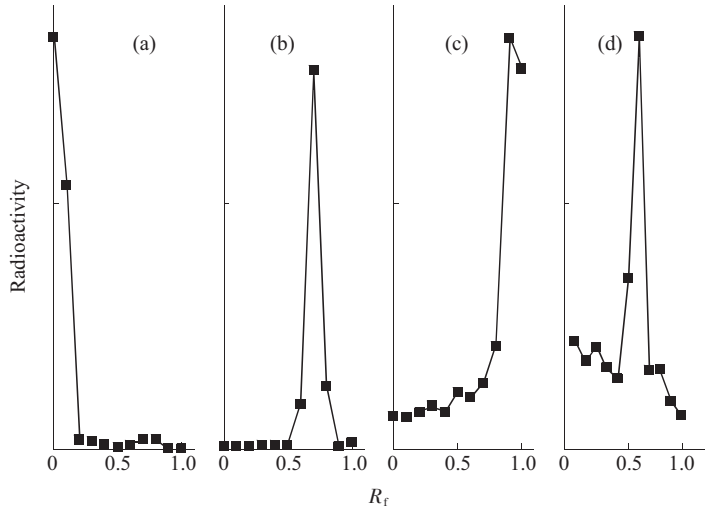

Fig. 1 Paper chromatograms of ^{99m}Tc-MAG3-phage (a), ^{99m}Tc-pertechnetate (b), ^{99m}Tc-MAG3 (c) and ^{99m}Tc-tartrate (d) in paper/saline system are shown

than 95% as indicated by the results of paper chromatography (Figure 1). The radiolabled phage was stable under the conditions of incubation, especially in serum. The labeled phage in paper/saline system remained at the origin (R_f = 0.0). Pertechnetate (R_f = 0.70), labeled tartrate (R_f = 0.5 ～0.6), and MAG3 (R_f = 0.9~1.0) in paper/saline system migrated.

2.2 ^{99m}Tc-MAG3-phage elimination in blood

Time course of ^{99m}Tc-MAG3-phage elimination in blood is shown in Figure 2. The clearance of ^{99m}Tc-labeled phage in blood was very fast from 5 min to 30 min and then slowed down. This verified that engineered phage had short serum half-life (12 ～ 18 min in different types of mice) [16]. In order to harvest more phage particles, the circulation of phage should be allowed *in vivo* for shorter time.

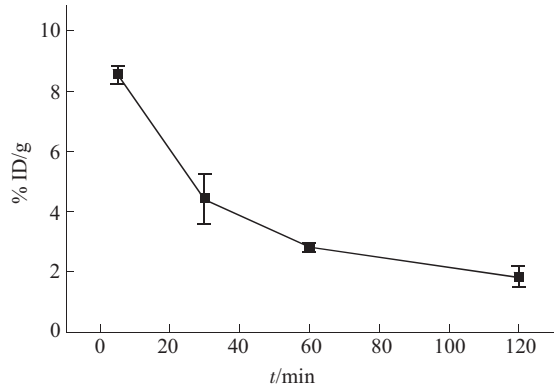

Fig. 2 Time course of phage elimination in blood is shown

2.3 Tissue uptake of ^{99m}Tc-MAG3-phage

The biodistribution of ^{99m}Tc-MAG3-phage in normal mice at different time points is shown in Table 1. Liver and spleen had the highest uptake, e.g.

Table 1 The biodistribution of ^{99m}Tc-MAG3-phage

%ID/g

Organ or tissue	Time			
	5 min	30 min	60 min	120 min
Liver	44.08 ± 3.17	33.52 ± 6.79	31.01 ± 4.13	25.41 ± 4.57
Spleen	30.93 ± 5.48	27.92 ± 6.74	26.14 ± 1.23	25.4 ± 8.30
Blood	8.52 ± 0.30	4.40 ± 0.84	3.13 ± 0.66	1.82 ± 0.34
Kidney	6.94 ± 1.29	5.22 ± 1.58	6.50 ± 1.61	3.99 ± 0.22
Lung	4.95 ± 0.87	4.33 ± 1.87	2.46 ± 0.19	1.84 ± 0.86
Stomach	3.93 ± 1.45	5.29 ± 1.86	3.76 ± 0.58	4.79 ± 0.46
Heart	2.56 ± 0.08	1.85 ± 0.55	1.28 ± 0.07	0.92 ± 0.03
Bone	1.80 ± 0.51	1.31 ± 0.45	2.98 ± 1.61	4.63 ± 1.99
Pancreas	1.31 ± 0.25	1.21 ± 0.47	0.69 ± 0.04	0.51 ± 0.13
S intestine	1.28 ± 0.12	1.18 ± 0.21	1.86 ± 0.19	3.97 ± 3.26
Muscle	1.07 ± 0.20	0.86 ± 0.10	0.69 ± 0.04	0.44 ± 0.07
L intestine	0.97 ± 0.11	1.41 ± 0.27	1.28 ± 0.05	1.94 ± 0.47
Brain	0.16 ± 0.00	0.18 ± 0.06	0.12 ± 0.01	0.07 ± 0.01

for ^{99m}Tc-MAG3-phage, was 44.08 %ID/g in liver and 30.93 % ID/g in spleen respectively at 5 min post injection. The accumulation in kidney, blood, lung and stomach was much lower than in liver or spleen, but higher than in other organs. The uptake of ^{99m}Tc-MAG3-phage in heart, muscle, pancreas and brain was markedly lower. There was little phage in brain at 2 h. In opposite to other tissues and organs, the radioactivity in stomach, intestine and bone gradually went up with time, although some fluctuations existed. These results were in consistence with results[16].

2.4 Extravasation of some phage particles to specific subendothelial tissues

Tissues/blood ratios of ^{99m}Tc-MAG3-phage at various points were depicted as Figure 3. If tissue/blood ratio is less than 1, it may be concluded that phage particles are primarily in blood at this time point according to reports [16, 22]. When ratio exceeds one, it may imply that the extravasation of some phage particles to subendothelial tissues does occur. At 5 min

post injection only liver/blood and spleen/ blood ratios exceeded 1 among the studied organs and tissues. At 0.5 h and 1 h post injection, kidney/blood and stomach/blood ratios exceeded 1 in addition to liver/blood and spleen/blood ratios. At 2 h post injection, liver/blood, kidney/blood, lung/blood, spleen/blood, stomach/blood, L intestine/blood, S intestine/blood and bone/blood ratios also exceeded 1. Interestingly, the ratios of heart/blood, pancreas/blood, muscle/blood and brain/blood were exclusively less than 1 at all four detecting time points from 5 min to 2 h, especially brain/blood ratios were quite low. Zou's result[16] only gave muscle/blood ratios that were also less than one until 24 h.

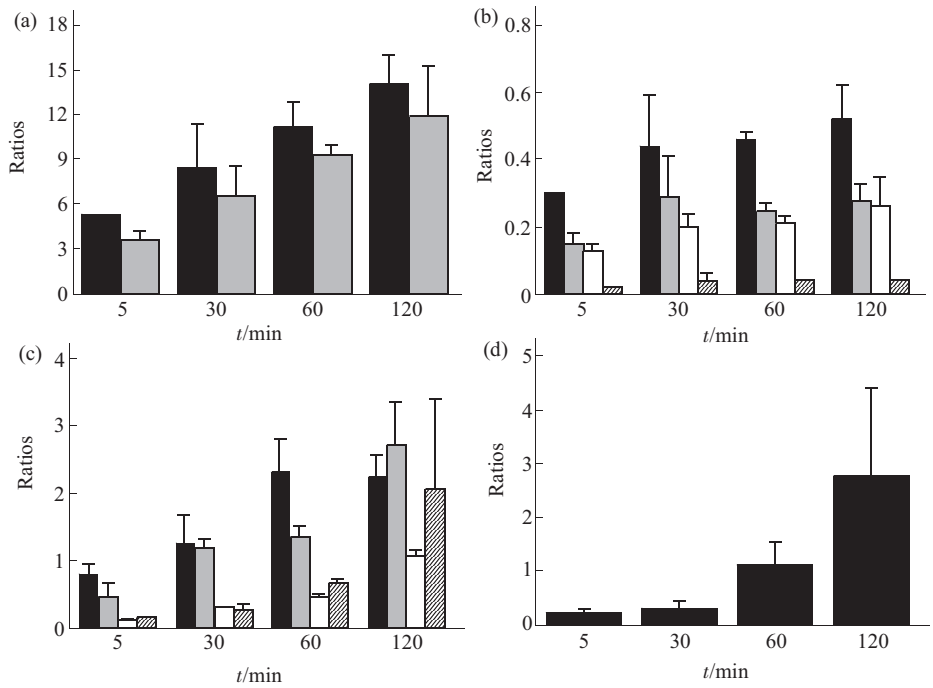

Fig. 3 The ratios of tissue to blood after injection of ^{99m}Tc-MAG3-phage are shown at various points

(a) ■: Liver/blood; ▒: Spleen/blood. (b) ■: Heart/blood; ▒: Pacreas/blood; □: Muscle/blood; ▨: Brain/blood. (c) ■: Kidney/blood; ▒: Stomach/blood; □: L int/blood; ▨: S int/blood. (d) ■: Bone/blood.

3 Discussion

Panning *in vivo* represents a significant new application of phage display technology. This panning method might improve the correlation between the binding characteristics of peptides and their *in vivo* tumor targeting properties. The most appropriate time for tissue collection may be determined according to the biodistribution of filamentous phage peptide library in mice. Because 5 min and 1 h were often chosen as circulation time *in vivo* in many reports, the biodistribution of ^{99m}Tc-labeled phage was studied from 5 min to 2 h. We found that there was a marked difference in radioactivity among different organs from 5 min to 2 h. This result lend further support to the notion that biodistribution of phage must be experimentally determined to optimize/facilitate discovery of organ or tumor cell specific-targeting phage and corresponding peptides[16, 20].

However, the activity of other organs or tissues was much less than activity in liver or spleen. Despite higher accumulation in kidney, but only (6.94±1.29)% ID/g at 5 min, it was far lower than either in liver or spleen. This was different from results in Zou's and Yip's reports[16, 20]. Although there was a little difference in the biodistribution of different tissues or organs in their reports by recovering phage, the difference was quite small. We thought different phage detecting methods resulted in such issues.

Zou *et al* found that tissues or organs/blood ratios were much less than one in all tissues and organs examined by 15 min. But we found that liver/blood and spleen/ blood ratios were greater than one at 5 min. Accumulation in heart, brain, muscle and pancreas was lower and tissue/blood ratios in these four organs were less than one by 2 h. It indicates that

phage particles hardly harbor these four organs and could not almost extravasate to these subendothelial tissues by 2 h. Therefore, peptides identified *in vivo* may target to intravascular receptors rather than subendothelial tissue receptors in these four organs by 2 h. But we could not easily conclude that phage had extravasated to these subendothelial tissues when intestine /blood ratios were more than one. Higher ratios may be caused by ^{99m}Tc from ^{99m}Tc-labeled phage degraded *in vivo* by target tissues.

In conclusion, the study provides a more accurate and rapid quantitative assessment of the biodistribution of filamentous phage peptide library to the design of panning experiment *in vivo*. The most appropriate time for tissue collection will be influenced both by the target organs or tissues and the specific location of target peptides. Because the clearance of ^{99m}Tc-labeled phage in blood was very fast from 5 min to 30 min, and the recovery of infectious phage particles decreased dramatically after 10 min or 30 min (the time was different for different organs) [21], the circulation time must not generally exceed 10 min, especially for the isolation of peptides against intravascular receptors. 30 min or longer panning time may prove superior for the isolation of target peptides against extravascular receptors. But phage in heart, pancreas, brain and muscle could not almost extravasate to these subendothelial tissues by 2 h. In addition, peptides identified at 5 min in liver and spleen should be further studied so as to determine them against intra- or extravascular targets. The results have important implications for future in panning protocols and for the circulation time of phage *in vivo*.

References

1 Smith G P. Filamentous fusion phase: novel expression vectors that display cloned antigens on the virion surface. Science, 1985, **228** (4705): 1315～1317

2 Azzazy H M E, Highsmith W E. Phage display technology: clinical applications and recent innovations. Clin Biochem, 2002, **35** (6): 425～445

3 Aina O H, Srok T C, Chen M L, *et al*. Therapeutic cancer targeting peptides. Biopolymers, 2002, **66** (3): 184～199

4 Arap M A. Phage display technology-Applications and innovations. Genet Mol Biol, 2005, **28** (1): 1～9

5 Zurita A J, Arap W, Pasqualini R. Maping tumor vascular diversity by screening phage display libraries. J Controlled Release, 2003, **91** (1～2): 183～186

6 Pasqualini R, Ruosiahti E. Organ targeting *in vivo* using phage display peptide library. Nature, 1996, **380** (6572): 364～366

7 Arap W, Kolonin M G, Trepel M, *et al*. Steps toward mapping the human vasculature by phage display. Nat Med, 2002, **8** (2): 121～127

8 Kolonin M G, Pasqualini R, Arap W. Molecular addresses in blood vessel as targets for therapy. Curr Opin Chem Biol, 2001, **5** (3): 308～313

9 Wang L F, Yu M. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr Drug Targets, 2004, **5** (1): 1～15

10 Oyama T, Sykes K F, Samli K N, *et al*. Isolation of lung tumor specific peptides from a random peptide library: generation of diagnostic and cell-targeting reagents. Cancer Letts, 2003, **202** (2): 219～230

11 Arap W, Pasqualini R, Ruoslaht E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science, 1998, **279** (5349): 377～380

12 Pasqualini R, Koivunen E, Ruoslahti E. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrians. J Cell Biol, 1995, **130** (5): 1189～1195

13 Kennel S J, Mirzadeh S, Hurst G B, *et al*. Labeling and distribution of linear peptides identified using *in vivo* phage display selection for tumors. Nucl Med Biol, 2000, **27** (8): 815～825

14 Smith G P, Petrenko V A. Phage display. Chem Rev, 1997, **97** (2): 391～410

15 Landon L A, Deutscher S L. Combinatorial discovery of tumor targeting peptides using phage display. J Cell Biochem, 2003, **90** (3): 509～517

16 Zou J, Dickerson M T, Owen N K, *et al*. Biodistribution of filamentous phage peptide libraries in mice. Mol Biol Reports, 2004, **31** (1): 121～129

17 Inchley C J. The activity of mouse Kupffer cells following intravenous inject of T4 bacteriophage. Clin Exp Immunol, 1969, **5** (1): 173～187

18 Geier M R, Trigg M E, Merril C R. Fate of bacteriophage lamda in non-immune germ-free mice. Nature, 1973, **246** (5430): 221～222

19 Ruschowski M, Gupa S, Liu G Z, *et al*. Investations of a 99mTc-labeled bacteriophage as a potential infection-specific imaging agent. J Nucl Med, 2004, **45** (7): 1201～1208

20 Yip Y L, Hawkins N J, Smith G, *et al*. Biodistribution of filamentous phage-Fab in nude mice. J Immunol Methods, 1999, **225** (1～2): 171～178

21 Molenaar T J M, Michon I, de Haas S A M, *et al*. Uptake and processing of modified bacteriophage M13 in mice: implication for phage display. Virology, 2002, **293** (10): 182～191

22 Winnard P, Chang F, Rusckowski M, *et al*. Preparation and use of NHS-MAG3 for technetium-99m labeling of DNA. Nucl Med Biol, 1997, **24** (5): 425～432

^{99m}Tc 标记丝状噬菌体肽库及其在正常小鼠体内的分布 *

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摘要 利用噬菌体展示技术已选出了多条与靶结合的肽. 然而，即使是体内直接筛选得到的，肽与肿瘤或靶器官的体内结合并不理想. 为了更好地理解噬菌体在体内的循环， 通过 MAG3 ^{99m}Tc 标记噬菌体肽库，研究了肽库在体内分布. 体内分布实验结果显示，^{99m}Tc- 噬菌体主要分布在肝和脾中，而心脏、肌肉、脑和胰腺这些器官或组织中的分布非常低. ^{99m}Tc- 噬菌体在胃、肠和骨中的累积，随着时间延长在不断升高，其他器官中的吸收则在不断降低. 从 5 min 到 30 min，^{99m}Tc- 噬菌体在血中清除迅速. 当噬菌体在体内循环足够长的时间后，一些噬菌体颗粒可以穿透血管进入并内化在器官或组织中. 总之，为了筛选具有高特异性和亲和性的肽，应该根据靶器官和筛选部位的不同，在筛选前确定合适的噬菌体在体内的循环时间.

关键词 丝状噬菌体肽库，生物分布，标记，^{99m}Tc

学科分类号 O615.42，Q785

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