Synthetic Peptides in the Form of Dendrimers Become Resistant to Protease Activity*

Received for publication, August 5, 2003, and in revised form, September 12, 2003
Published, JBC Papers in Press, September 12, 2003, DOI 10.1074/jbc.M308615200

Luisa Bracci‡, Chiara Falciani‡, Barbara Lelli‡, Luisa Lozzi‡, Ylenia Runci‡, Alessandro Pini‡, Maria Grazia De Montis¶, Alessandro Tagliamonte§, and Paolo Neri‡

From the ‡Department of Molecular Biology, Laboratory of Biochemistry and Molecular Biology, University of Siena, Via Fiorentina, 1 and the ¶Department of Neuroscience, Pharmacology Unit, University of Siena, Via A. Moro, 4, 53100 Siena, Italy

In previous papers, we observed that dendrimers of peptide mimotopes of the nicotinic receptor ligand site are strong antidotes against the lethality of the nicotinic receptor ligand a-bungarotoxin. Although their in vivo activity is identical to that of dendrimers, the corresponding monomeric peptide mimotopes are not effective in vitro. Because the higher in vivo efficiency of dendrimers could not in this case be related to polyvalent interaction, the stability to blood protease activity of monomeric versus tetrabranched dendrimeric mimotope peptides was compared here by incubating three different mimotopes with human plasma and serum. Unmodified peptides and cleaved sequences were followed by high pressure liquid chromatography and mass spectrometry. Tetrabranched peptides were shown to be much more stable in plasma and also in serum. To assess the notable stability of multimeric peptides, different bioactive neuropeptides, including enkephalins, neurotensin and nociceptin, were synthesized in monomeric and tetrabranched forms and incubated with human plasma and serum and with rat brain membrane extracts. All the tetrabranched neuropeptides fully retained biological activity and generally showed much greater stability to blood and brain protease activity. Some tetrabranched peptides were also resistant to trypsin and chymotrypsin. Our findings provide new insights into the possible therapeutic use of bioactive peptides.

Hundreds of peptides with potential therapeutic activities have been identified. These include naturally occurring peptide hormones and neurotransmitters, which influence and control series of vital functions, such as cell proliferation, tissue development, metabolism, immune defense, perception of pain, reproduction, behavior, and blood pressure. Selective agonists or antagonists of these natural peptides are extremely useful for the investigation of peptidergic systems and are also potential therapeutic agents (1). Moreover, several peptide fragments or mimotopes derived from potential therapeutic proteins show promising biological activity (2).

However, the use of peptides as therapeutic drugs has largely been limited by their short half-life in vivo. Because peptides are mainly broken down by proteases and peptidases, peptide delivery is the bottleneck in the development of new peptide drugs. To increase peptide half-life, many strategies involving different levels of chemical modification are possible (3, 4). The introduction of D-amino acids, or pseudo amino acids, and peptide cyclization are the most common strategies to increase peptide stability. However, these modifications may profoundly alter peptide activity. Alternatively, peptidomimetic molecules can be developed by the synthesis of conformationally restricted compounds, in which the peptide is locally or globally constrained in order to reproduce the active conformation. The resulting structures are mostly non-peptide molecules, more resistant to degrading enzymes.

In general, peptide molecules have the advantage of good specificity and high selectivity and the disadvantage of poor metabolic stability and limited distribution. Although non-peptide molecules have better stability and pharmacokinetics, they may have very different specificity and affinity to natural peptides. The design and synthesis of new peptidomimetic compounds therefore involve careful testing of their selectivity, specificity, and affinity for target biomolecules.

In this study we report results suggesting that synthesis of bioactive peptides in multiple antigen peptide (MAP)1 dendrimeric form can result in increased half-life, due to acquired resistance to protease and peptidase activity. MAPs have a peptidyl core of radially branched lysine residues onto which peptides can be added using standard solid-phase chemistry (5). MAPs can be more efficient than monomeric peptides in diagnostic applications. Moreover, they also proved to be good immunogens against a number of pathogens (6). The in vitro and in vivo efficiency of dendrimeric peptides like MAPs is generally ascribed to their multimeric nature, which enables polyvalent interactions.

We reported previously that the tetrameric MAP form of the peptide mimotope p6.7 and its high affinity analogues pDD and pDDD, which compete with the nicotinic receptor (nAchR) to bind the snake neurotoxin a-bungarotoxin (a-bgt), efficiently neutralize toxin lethality in mice, unlike the corresponding monomeric sequences. This happens despite the nearly identical affinity constant (Kd) and half-maximal inhibition concentration (IC50) of MAPs and monomeric peptides, and their similar in vitro activity (7, 8). Because in the particular case of peptide ligands of a monomeric soluble toxin, MAPs have no polyvalent interaction-associated advantage with respect to

* This work was supported by grants (to L. B.) by Italian Ministry of Education, University and Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-0577-234918; Fax: 39-0577-234903; E-mail: bracci@unisi.it.

1 The abbreviations used are: MAP, multiple antigen peptide; nAchR, nicotinic acetylcholine receptor; a-bgt, a-bungarotoxin; M-enk, Met-enkephalin; NT, neurotensin; NC, nociceptin; MS, mass spectrometry; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IAA, iodoacetamide; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; L-enk, Leu-enkephalin; Fmoc, N-(9-fluorenylmethoxycarbonyl).

This paper is available on line at http://www.jbc.org
monomeric sequences (which was confirmed by results of in vitro competition experiments), we concluded that the notable in vivo efficiency of MAPs seemed to be related to a pharmacokinetic advantage, such as different clearance, or resistance to peptidase and protease activity.

In order to verify this possibility, we examined here the stability of the monomeric and MAP mimotopes in human plasma and serum. Moreover, we synthesized several tetrameric MAPs, reproducing sequences of different bioactive peptides, and we compared their resistance to proteases to that of the corresponding monomeric peptides.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Solid-phase synthesis was carried out on a MultiSynTech Syro automated multiple peptide synthesizer (Witten, Germany), employing N-(9-fluorenylethoxycarbonyl) (Fmoc) chemistry with 2-(4H-benzo triazole-1-yl)-1,1,1,3,3,3-tetramethyluronium hexafluorophosphate/N,N-diisopropylaminolamine activation. Side chain protecting groups were trityl for His, 2,2,4,6,7-pentamethyldi hydrobenzofuran-5-sulfonyl for Arg, tert-butyl ether for Ser and Tyr, tert-butyl ester for Asp and Glu, and tert-butyloxycarbonyl for Trp. Monomeric peptides HRYYESSLPWPFD (p6.7), FRYYESSLPWDP (pDD), FRYYESLWPDD (pDDD), YGFM (Met-enkephalin, M-enk), YGFL (Leu-enkephalin, L-enk), ELYENKPRRPYIL (neurotensin, NT), [3H]nociceptin, (PerkinElmer Life Sciences). Nonspecific binding was determined in the presence of 10 μM [N-F[5]hociceptin fragment 1-13-amide (Sigma). All experiments were performed in ice-cold 50 mM Tris-HCl, 2 mg/ml BSA, 10 mM EDTA, 10 mM EGTA, 10 mM aminobenzamidine, 10 mM IAA, and 10 mg/liter soybean trypsin inhibitor, pH 7.4. The IC50 of the peptides was evaluated by non-linear regression analysis of curves using GraphPad Prism 3.02 software.

**Inhibition of Ligand Binding to Rat Brain Membranes**—Monomeric and tetrameric enkephalins, diluted at concentration ranging from 10-5 to 5 × 10-8 M in ice-cold 50 mM Tris-HCl buffer pH 7.4, were incubated with 15 mg/ml rat brain membranes in the presence of 0.5 nM [3H]naloxone. After 1 h at 37 °C, the reaction was stopped by adding 4 ml of ice-cold Tris-HCl buffer with 0.5 mM EDTA. The membranes were then filtered through Whatman GF/B filters, detergent treated with 0.1% polyethyleneimine, and rinsed twice with 4 ml of the same buffer using a Buchner Cell Harvester. [3H]Naloxone binding was detected by a β-counter. Nonspecific binding was determined in the presence of 10-6 M naloxone.

For experiments performed in the absence of protease activity, all samples were diluted in 50 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 10 mM aminobenzamidine, 10 mM IAA, and 10 mg/liter soybean trypsin inhibitor, pH 7.4. The IC50 of the peptides was evaluated by non-linear regression analysis of curves using GraphPad Prism 3.02 software.

Monomeric and multimeric nociceptin, diluted at concentrations ranging from 10-5 to 10-12 M, were incubated with 15 mg/ml rat brain membranes in the presence of 1 nM [3H]nociceptin (PerkinElmer Life Sciences). Nonspecific binding was determined in the presence of 10-5 M N[5]-Enkephalin fragment 1-13-amide (Sigma). All experiments were performed in ice-cold 50 mM Tris-HCl, 2 mg/ml BSA, 10 mM EDTA, 10 mM EGTA, 10 mM aminobenzamidine, 10 mM IAA, and 10 mg/liter soybean trypsin inhibitor, pH 7.4. After centrifugation for 40 min at 40,000 × g at 4 °C, the pellet was washed, centrifuged again under the same conditions, resuspended in the same buffer, and sonicated to avoid membrane aggregation. HT 29 cell membranes were prepared fresh upon use.

Monomeric and multimeric neurotensin were incubated with 0.5 mg/ml HT 29 cell membranes diluted in ice-cold Tris-HCl, 2 mg/ml BSA, 10 mM EDTA, 10 mM EGTA, 10 mM aminobenzamidine, 10 mM IAA, 10 mg/liter soybean trypsin inhibitor, pH 7.4, at concentrations ranging from 10-5 to 10-12 M in the presence of 1 nM [3H]Neurotensin (PerkinElmer Life Sciences). Nonspecific binding was determined in the presence of 10-5 M neurotensin (Sigma).

**RESULTS**

**Stability of Dendrimeric Mimotopes to Blood Proteases**—We previously produced synthetic peptides mimicking the snake neurotoxin-binding site of the nicotinic receptor, and we found that the in vivo efficiency of these mimotopes reflects their affinity and IC50 value only when they are synthesized in MAP form (7, 8). The efficacy of the tetrameric peptides in vivo could not be ascribed to any kinetic or thermodynamic effect and therefore seemed related to differences in pharmacokinetic behavior.

To verify this possibility, we compared the resistance of monomeric and tetrabranched peptide mimotopes in human plasma and serum. Monomeric and tetrabranched peptides were incubated with human plasma or serum for 2 or 24 h, and the mixture was analyzed by HPLC and mass spectrometry to follow the presence of uncleaved monomeric and MAP peptides. As a general rule, peptides that were cleaved in plasma in 2 h were also cleaved in serum in the same time. Those resistant in serum after 24 h were also resistant in plasma (Table I).

Monomeric peptide mimotopes (p6.7, pDD, and pDDD) were completely degraded within 2 h in serum. Fragments derived from proteolytic cleavage were detected by MS in the HPLC-eluted material, after 2 h of incubation in plasma. Conversely, tetrabranched forms of the same peptides were still detected after 24 h in plasma and serum (Fig. 1). Incubation for 2 h in...
human plasma or serum increased the IC_{50} value of monomeric peptides by at least 3 log, whereas MAP peptides retained their inhibition power after 24 h (Fig. 2).

**Stability of Dendrimeric Neuropeptides to Blood and Brain Proteases**—In order to verify whether the enhanced stability in plasma and serum could be considered a general behavior of tetrabranched peptides, we synthesized the monomeric and the tetrmeric MAP form of different natural bioactive peptides in order to compare their resistance to protease activity. We chose Met-enkephalin, Leu-enkephalin, neurotensin, and nociceptin because they have very interesting pharmacological properties but very short half-lives in vivo. Moreover, this choice allowed testing peptides of different length and sequence, going from 5 residues of enkephalins to 17 residues of nociceptin.

**Enkephalins**—Enkephalins are endogenous ligands of opioid receptors. Analogues of enkephalins are very well known and its derivatives) or pseudo-peptide molecules including pseudo or D-amino acids (9). In this work, we synthesized and tested the natural peptide sequence of Met- and Leu-enkephalins. The MAP forms of M-enk and L-enk were analyzed for their ability to bind opioid receptors by testing inhibition of [3H]naloxone binding to rat brain cell membranes. MAPs of M- and L-enk have an IC_{50} that is one-tenth that of their monomeric counterparts (Fig. 3). In the presence of protease inhibitors, the IC_{50} value of tetrmeric and monomeric enkephalins was nearly identical (3.7 \times 10^{-7} \text{M} for M-enk and MAP M-enk, respectively, and 5.7 \times 10^{-7} \text{M} for L-enk and MAP L-enk, respectively). When MAP and monomeric enkephalins resistance was tested in plasma and serum by HPLC and MS, the latter were still present after 2 h in plasma and were found to be cleaved within 2 h in serum, whereas their tetrabranched analogues were still present after 24 h of incubation in plasma (Table I).
Resistance to protease activity was also tested by incubating tetrameric and monomeric peptides with rat brain membrane extracts. Monomeric Met- and Leu-enkephalins were found to be degraded within 24 h, whereas their multimeric analogues were still detectable.

Neurotensin—NT is a 13-amino acid peptide originally isolated from calf hypothalamus (10). It has the dual function of neurotransmitter or neuromodulator in the nervous system and local hormone in the periphery. NT is a neuromodulator of dopamine transmission and anterior pituitary hormone secretion and exerts potent hypothalamic and analgesic effects in the brain (11). In the periphery, NT is a paracrine and endocrine modulator of the digestive tract and cardiovascular system. NT receptors are overexpressed in a number of neuroendocrine human tumors (12). Interest in NT is related to the possibility of developing antipsychotic (11) and anticancer (12, 13) drugs.

We synthesized monomeric and tetrabranched neurotensin and its short analogue NT-(8–13) (14). Bioactivity of monomeric and MAP peptides was checked by testing the inhibition of nM [3H]neurotensin-specific binding to membranes prepared from the human colon adenocarcinoma cell line HT 29 (15). Experiments were performed in the presence of protease inhibitors. MAP NT efficiently inhibited nM [3H]neurotensin-specific binding, and its IC50 value was analogous to that of monomeric NT (Fig. 4). NT-(8–13) was more effective than whole NT. MAP NT-(8–13) had an IC50 of 1.9 × 10–9 M, which is about 1 log lower than that of monomeric NT-(8–13) and more than 2 log lower than NT and MAP NT.

The resistance to protease activity in human plasma and serum was tested for the four peptides. We found that the MAP form was more resistant than the monomeric form. MAP forms of NT and NT-(8–13) were still detectable in serum after 24 h, when the monomeric forms had disappeared (Table I).

When the tetrameric and monomeric forms of NT and NT-(8–13) were incubated in rat brain membrane extracts, monomeric peptides were present after 2 h, but many by-products appeared at 24 h, whereas the MAP analogues resisted beyond 24 h.

Nociceptin—NC (16, 17) is an endogenous heptadecapeptide that displays nanomolar affinity for the opioid receptor-like ORL1, a G protein-coupled receptor. NC modulates nociceptive and locomotor behavior in mice (18).

We synthesized the monomeric NC and tetrameric MAP NC and tested their behavior upon plasma and serum treatment. Monomeric NC was degraded within 2 h in serum and plasma, whereas MAP NC was still detectable after 24 h in plasma.

NC and MAP NC were incubated with rat brain membrane extracts, and while the monomeric peptide disappeared within 24 h, MAP NC was still recoverable.

Bioactivity of monomeric and MAP NC was assessed by testing the inhibition of [3H]nociceptin-specific binding to rat brain membranes in the presence of protease inhibitors. MAP NC inhibited the binding of the labeled peptide with an IC50 of 1.9 × 10–9 M, whereas the IC50 of the monomer was 8.6 × 10–9 M (Fig. 5).

Stability of Dendrimeric Peptides to Trypsin and Chymotrypsin—The increased stability of dendrimeric peptides was also confirmed with purified proteases. Tetrabranched mimotopes...
MAP p6.7, MAP pDD, and MAP pDDD were not cleaved by trypsin in 24 h, whereas their corresponding monomers were proteolyzed within 2 h, except for peptide p6.7, which was still detectable. Most monomeric and MAP peptide mimotopes were degraded by chymotrypsin after 2 h of incubation. However, the MAP form of the peptide pDDD was still found after 24 h of incubation.

Monomeric M- and L-enk were cleaved by chymotrypsin but not by trypsin. Their multimeric analogues were detectable after exposure to chymotrypsin for 2 h.

NT was degraded by chymotrypsin within 2 h and by trypsin within 24 h when its tetrabranched analogue was still present. Moreover, the bioactive analogue NT-(8–13) in its tetrabranched form showed remarkable resistance to the purified enzymes; it remained intact after 24 h of incubation in trypsin and chymotrypsin, whereas its monomeric analogue only resisted for 2 h with trypsin and less with chymotrypsin.

NC is a substrate for chymotrypsin (degraded in 2 h) but not for trypsin; however, the MAP analogue MAP NC was stable in chymotrypsin even after 24 h (Table I).

**DISCUSSION**

In this paper we provide data indicating that synthesis of different peptide sequences, including natural endogenous peptides, in a MAP dendrimeric form allows retaining full peptide biological activity and results in a dramatic increase in peptide stability to blood and brain protease and peptidase activity.

The higher stability of MAP forms to protease activity could be a consequence of their unnatural branched peptide structure. Moreover, in the case of short peptides, the activity of specific peptidases (like many membrane, blood, or central nervous system metallo-oligopeptidases) (19), which are selectively active on short sequences, could be prevented by the mass increase and steric hindrance of MAPs. This possibility seems to be supported by the reported results, indicating a higher general stability of MAPs to blood and brain proteases than to trypsin and chymotrypsin.

Synthetic peptides composed exclusively of natural amino acids, but having a longer half-life than natural peptides, may have major applications as therapeutic drugs. For example, the MAP enkephalins we produced and tested here consist of the same residues as natural enkephalins and have the same binding affinity in vitro. MAP enkephalins might have an improved activity related to greater stability and not to higher receptor affinity. This could be an advantage for pharmacological use as they may avoid triggering immune response, while having less intense morphine-like side effects related to overstimulation of receptors.

Another important possible development of the protease-resistant MAPs described here is the application of neurotensin to tumor therapy and in vivo imaging. The finding of new specifically targeted radiopharmaceuticals is an important challenge in cancer research, where tumor-specific small peptides may have major potential applications, provided that they are stabilized to overcome their degradation by proteases and peptidases. Currently, analogues of NT and particularly of NT-(8–13) are under investigation to target a variety of neuroendocrine human tumors overexpressing NT receptors (12–13, 20). We demonstrated here that NT and NT-(8–13), once...
synthesized in a MAP form, fully retain their biological activity and become much more resistant to blood proteases. Moreover, the tetrabranched MAP of peptide NT-(8–13) has a 10-fold better IC50 value than the corresponding monomeric peptide. These dendrimeric peptides are therefore candidates for the development of new specific tumor-targeted biomolecules.

Although the possible higher stability of dendrimeric peptides has been speculated previously (6), to our knowledge this is the first demonstration of MAPs resistance to blood and brain proteases. A higher stability of MAP activity following trypsin treatment, with respect to the corresponding linear peptide, was already reported for an antimicrobial peptide (21), although no chemical characterization of peptide proteolysis was reported. MAP stability was deduced from the higher residual antimicrobial activity after incubation with the enzyme.

Dendrimeric peptides were reported to have a higher in vivo efficiency with respect to corresponding monomeric sequences in several cases (6, 22), but in general they were used in conditions where they could bind through multivalent interactions. This effect can somehow have masked the pharmacokinetic advantage of dendrimeric peptides or can at least have rendered their different in vivo stability not immediately evident. In the particular case of dendrimeric mimotopes that bind a soluble monomeric ligand and that have identical in vitro activity with respect to corresponding monomers, it was clear that the in vivo efficiency of dendrimeric peptides could only be related to a different pharmacokinetic behavior (7–8). This enabled us to distinguish between kinetic and pharmacokinetic advantage of dendrimers in vivo.

In conclusion, our results indicate that synthesis in dendrimeric form may be a general method to increase in vivo stability, and consequently half-life, of bioactive peptides.

The biological activity of a peptide might be lost in the MAP derivative, and tests on this feature obviously should be carried out. In the cases analyzed here, the MAP forms retained the full activity of native peptides, both when peptides bind to membrane receptors, like enkephalins, neurotensin, and nociceptin, and when they bind soluble proteins, like peptide mimotopes of a receptor-binding site. Moreover, biological activity and stability to protease activity was retained in dendrimers of peptides having different sequence and different length.

REFERENCES

1. Hruby, V. J. (2002) Nat. Rev. Drug Discov. 1, 847–858
2. Melsen, R. H., Pujik, W. C., and Slootstra, J. W. (2000) J. Mol. Recognit. 1, 352–359
3. Adessi, C., and Soto, C. (2002) Curr. Med. Chem. 9, 963–978
4. Goodman, M., Zapf, C., and Rew, Y. (2001) Biopolymers 60, 229–245
5. Tam, J. P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5409–5413
6. Sadler, K., and Tam, J. P. (2002) Rev. Mol. Biotechnol. 90, 195–229
7. Braazi, L., Lozzi, L., Pini, A., Lelli, B., Falciani, C., Nicolai, N., Bernini, A., Spreatfo, A., Söldani, P., and Neri, P. (2002) Biochemistry 41, 10194–10199
8. Lozzi, L., Lelli, B., Runci, Y., Scali, S., Bernini, A., Falciani, C., Pini, A., Nicolai, N., Neri, P., and Braazi, L. (2003) Chem. Biol. 10, 411–417
9. Dooley, C. T., and Houghten, R. A. (1999) Biopolymers 51, 379–390
10. Carraway, R., and Leeman, S. E. (1973) J. Biol. Chem. 248, 6854–6861
11. Binder, E. B., Kinkead, B., Owens, M. J., and Nemeroff, C. B. (2001) Pharmacol. Rev. 53, 453–586
12. Garcia-Garayoa, E., Alleman-Tannahill, L., Blauenstein, P., Wüllmann, M., Carrel-Reny, N., Tourwe, D., Iberbeke, K., Conrath, P., and Schüleger, P. A. (2001) Nucl. Med. Biol. 28, 75–84
13. Hillairet de nicefaron, M., Baguin, O., Thiéyelin, C., Dussaillant, M., Rostene, W., Barbet, A., and Gruanz-Guyon A. (2002) Bioconjugate Chem. 13, 854–862
14. Hong, P., Zaidi, J., Cusack, B., and Richelson, E. (2002) Bioorg. Med. Chem. 10, 3849–3858
15. Guly, D., Labeeuw, B., Boigegrain, R., Oury-Donat, F., Bachy, A., Poncelet, M., Steinberg, R., Suad-Chagnay, M. F., Santucci, V., Vita, N., Pecceu, P., Labbe-Jullie, C., Kitabgi, P., Soubrie, P., Le Fur, G., and Mafrand, J. P. (1997). J. Pharmacol. Exp. Ther. 280, 802–812
16. Meunier, J. C., Mollereau, C., Toll, L., Suad-Chagnay, M. F., Moisand, C., Alvinerie, P., Butot, J. L., Guillouet, J. C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., and Costentin, J. (1995) Nature 377, 532–535
17. Reinscheid, R. K., Nothacker, H. P., Bourson, A., Arditi, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J., and Civelli, O. (1995) Science 269, 792–794
18. Mogil, J. S., and Pasternak, G. W. (2001) Pharmacol. Rev. 53, 381–415
19. Shrimpton, C. N., Smith, A. I., and Lew, R. A. (2002) Bioorg. Med. Chem. Biol. 9, 5409–5413
20. Brauelhmeier, M., Garcia Garayoa, E., Blanc, A., Holzer, B., Gergely, S., Tourwe, D., Schüleger, P. A., and Blauenstein, P. (2002) Nucl. Med. Biol. 29, 321–327
21. Tam, J. P., Lo, Y. A., and Jiang, J. L. (2002) Eur. J. Biochem. 269, 923–932
22. Mourez, M., Kane, R. S., Mogridge, J., Metcalfe, S., Deschatelates, P., Sellam, B. R., Whitesides, G. M., and Collier, R. J. (2001) Nat. Biotechnol. 19, 958–961
