Chapter 3

Polyamide-Peptide and Polyamide-Small Molecule Conjugates for Cellular Uptake Studies

Chapter 3A

Polyamide-Peptide Conjugates for Cellular Uptake Studies

Abstract

In order that polyamides or polyamide-peptide conjugates be useful as diagnostic or therapeutic tools in living organisms, they must be able to cross the cellular membrane of cells within target tissues and transit to the nuclei of those cells. In proof-of-principle *in vitro* experiments polyamides were shown to inhibit the transcription of target genes, presumably by competing against natural transcription factors for promoter binding sites. In other *in vitro* experiments polyamide-peptide conjugates were shown to activate transcription of target genes, presumably by recruitment of the RNA polymerase II holoenzyme to promoter regions. Attempts to duplicate these successes in living model systems met with limited success.

It was postulated that the difficulty in transferring successful *in vitro* results to living systems lay in the inability of polyamides to cross cell membranes. In order to overcome this challenge, polyamides were conjugated to peptides shown to increase cellular uptake and to small molecules that might be recognized by cellular receptors, facilitating polyamide influx into the cell. Polyamides were also added to cells in growth medium containing drugs shown to induce cellular uptake of certain compounds. Though meeting with little initial success, these attempts increased the body of knowledge of how polyamide conjugates interact with DNA, how they interact with cells, and laid the groundwork for more successful experiments documented in Chapter 4.

Introduction

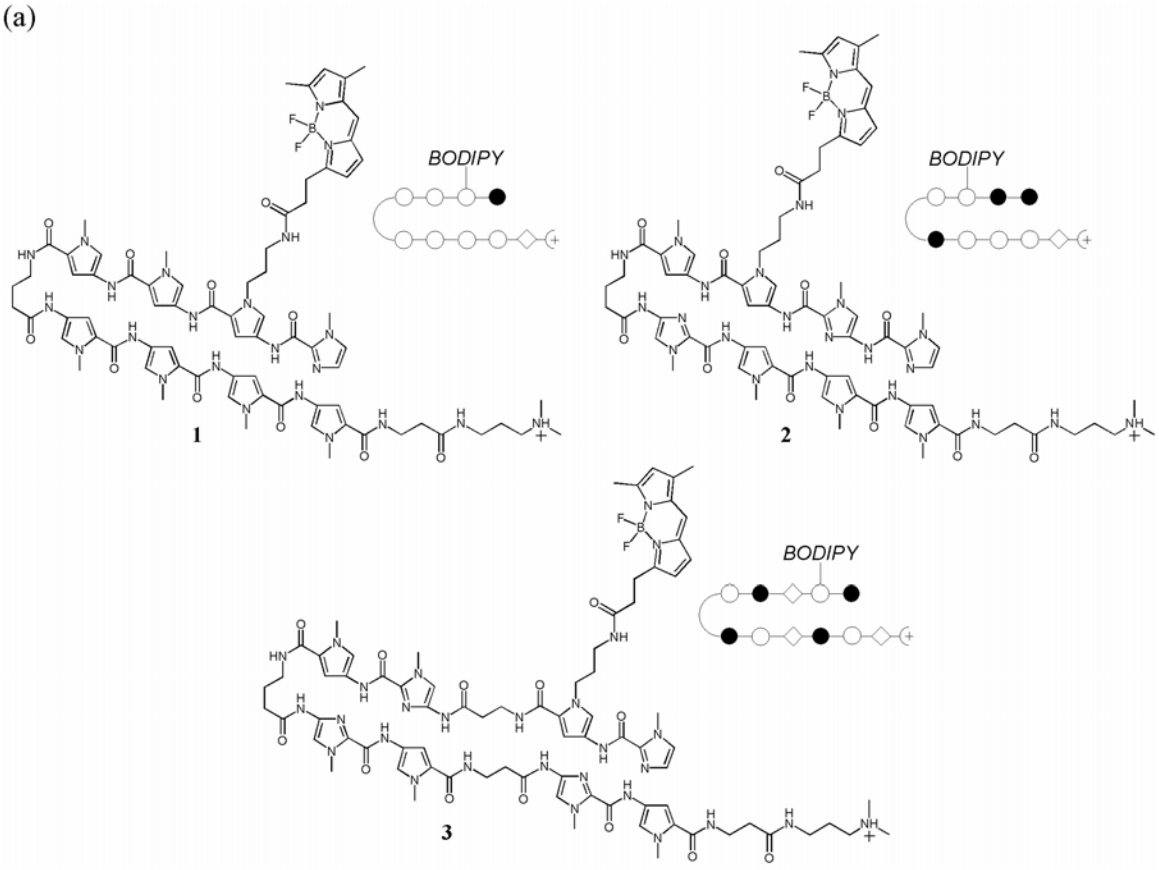
Polyamides are inherently interesting as probes of DNA recognition, due to the modularity of their design and their resulting ability to target nucleic acid sequences in a programmed manner. These molecular tools have been used effectively in several *in vitro* systems to regulate gene processing. As inhibitors of transcription, polyamides have been quite effective in a number of model systems (Figure 1.8). Once conjugated to activation peptides, polyamides have also functioned as transcriptional activators (Chapter 2). Ultimately it is desirable that polyamides have the capacity to affect the transcriptional machinery in cell cultures and living organisms. Studies with the human immunodeficiency virus (HIV) promoter and inhibition of viral replication provided early examples of the successful application of polyamides as potent regulators in primary human lymphocytes[1], as well as in live-cell models.[2] Polyamides were also effective in causing gain- or loss-of-function phenotypes when fed to *Drosophila melanogaster*.[3,4]

Attempts to inhibit the transcription of endogenous genes in cell lines other than insect or T-lymphocytes have met with little success. For example, polyamides targeted to the Ets binding site of the HER2/neu promoter were shown to inhibit binding of the transcription factor ESX at nanomolar concentrations and to inhibit transcription of the HER2/neu gene in cell-free experiments.[5] However, those polyamides display no activity in HER2-overexpressing SKBR-3 cells.[6] In an attempt to determine the reason why no inhibition of transcription was observed in the cell-based assay, fluorescent conjugates of polyamides attached to the Bodipy FL fluorophore were synthesized. These compounds were then applied to SKBR-3 cells and imaged by laser scanning confocal microscopy.

The polyamide conjugates were not seen to localize in the nucleus of the living cells, rather they were sequestered in the cytoplasm.[6]

In order to more generally determine the cellular localization characteristics of polyamides, a small series of polyamide-Bodipy conjugates were synthesized, applied to a variety of cell lines, and imaged by confocal microscopy (Figure 3.1).[7] With the

(a)



(b)

Conjugate	SKBR-3: human breast cancer	CEM: human cultured T-cells	T-cells: human primary T-cells	NB4: human leukemia	293: human kidney fibroblast	Sf9: fall armyworm (insect)	Kc: Drosophila (insect)
1	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
2	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
3	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm

Figure 3.1 Localization of polyamide–Bodipy conjugates in live cells as determined by confocal microscopy. (a) Chemical structures and ball-and-stick models of polyamide-Bodipy conjugates. (b) The designation 'nucleus' indicates observation of fluorescence in the interior of the nucleus. The designation 'cytoplasm' indicates cellular, non-nuclear fluorescence. Cells were imaged directly following 20 h incubation with 5 μM **1**–**3** under normal growth conditions for each cell-line.[7]

exception of human T-cells and T-cell-derived cell lines, polyamide conjugates **1-3** localized in the cytoplasm of all cells tested (Figure 3.2). This result suggested that transcriptional regulation experiments occurring in T-cells or T-cell-derived lines would not be negatively influenced by the cellular uptake of polyamides, while those in other cell lines were prohibited from showing positive results due to poor polyamide uptake.

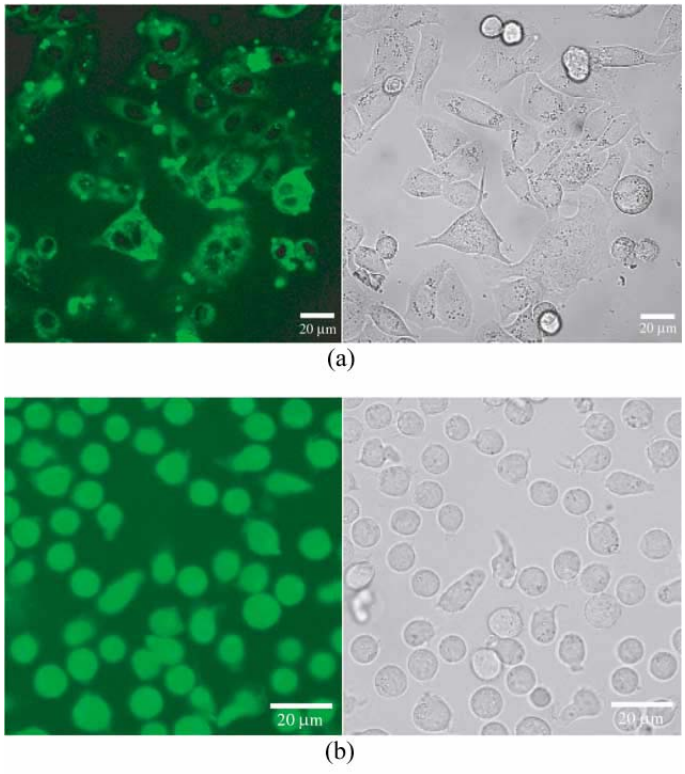


Figure 3.2 Localization of **1** in live human cell lines. (a) Conjugate **1** in live SKBR-3 cells, showing cytoplasmic staining. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm. (b) Conjugate **1** in live CEM cells, showing nuclear staining. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm.[7]

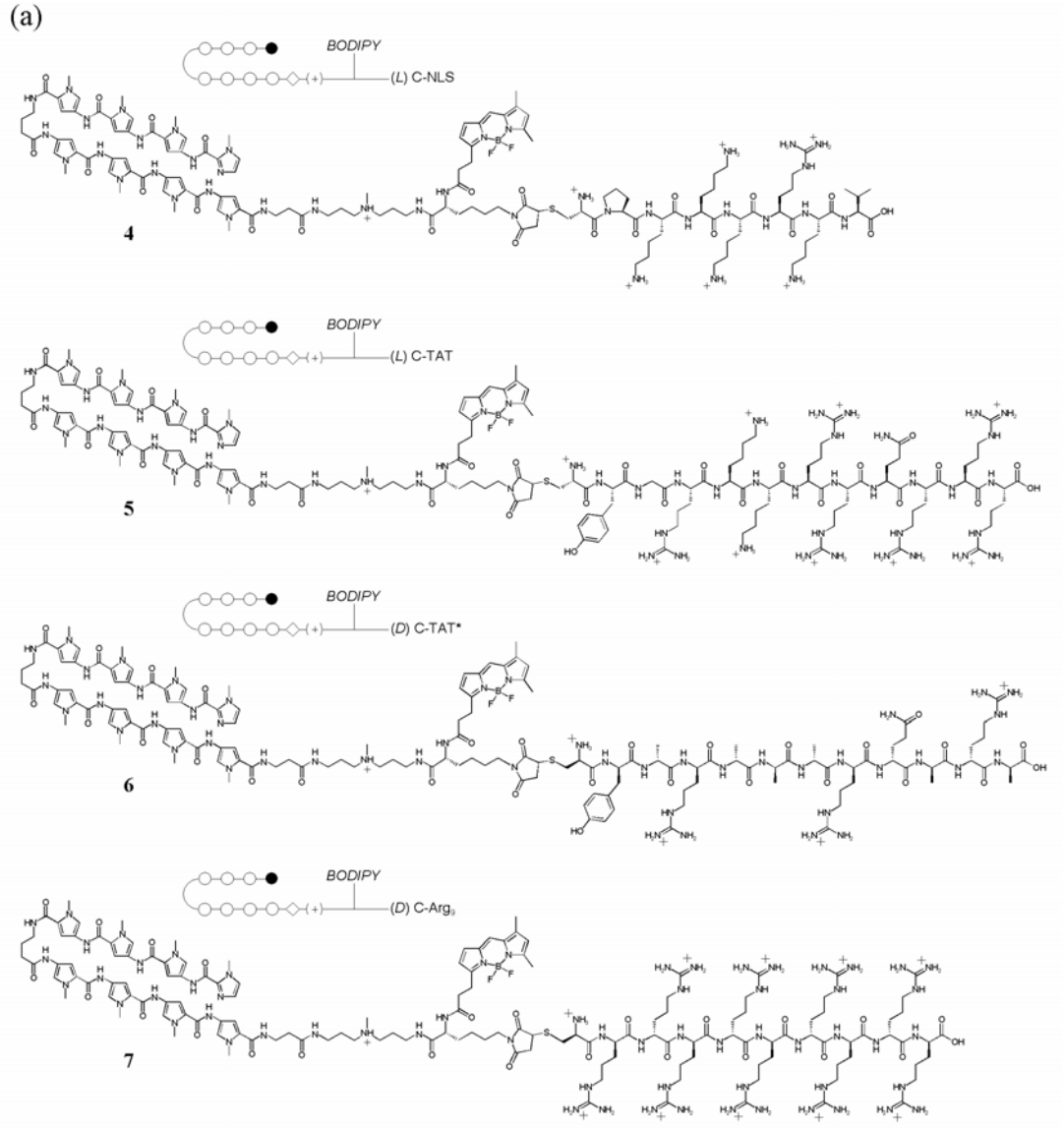
In order to overcome cytoplasmic sequestration of polyamide-fluorophore conjugates, polyamides bearing both a Bodipy fluorophore and one of several carrier peptides were synthesized. Carrier peptides have been used to transport small molecules, proteins, and oligonucleotides into both cells and nuclei.[8] Two of the most widely-

studied carrier peptides are TAT,[9-12] a basic domain derived from the HIV-1 Tat protein,[13] and the simian vacuolating virus-40 (SV-40) nuclear localization sequence (NLS) peptide,[14-17] derived from a basic region from the SV40 T-antigen protein.[18] The NLS is known to be recognized by a specific membrane protein receptor, karyopherin α.[18,19] The mechanism of TAT transduction is unknown, appearing not to involve receptor-, transporter-, endosome-, or adsorptive-endocytosis-mediated processes.[8,20] Since TAT-mediated uptake bypasses known endosomal routes, TAT is known to be a nuclear localization signal,[21] and since polyamide-Bodipy conjugates localize to the cytoplasm in discrete spots, possibly endosomes, TAT-polyamide-Bodipy conjugates should be expected to transit to the nucleus.

Several synthetic derivatives of TAT showing increased ability to facilitate uptake have been developed. Polyarginine peptide Arg_9 was created by Wender and co-workers as an analog of TAT. It was shown that (*D*) Arg_9 outperformed TAT in terms of uptake into Jurkat cells.[22] TAT* was developed by Dowdy and co-workers through alanine scanning to be a helical mimetic of TAT bearing a minimum number of arginine residues.[23] TAT* peptides composed of both *L*- and *D*-amino acids have been shown to be active uptake carriers. Peptides composed of *D*-amino acids are expected to have greater stability to proteolytic degradation than their natural counterparts of *L*-chirality.

Carrier peptide-polyamide-Bodipy conjugates **4**-**7** (Figure 3.3a) were synthesized and tested for their ability to stain the nuclei of several cell lines. TAT-polyamide-Bodipy and (*D*) Arg_9-polyamide-Bodipy conjugates **5** and **7** were observed to stain the nuclei of most cell lines tested, while NLS- and (*D*) TAT*-conjugates **4** and **6** generally were found to localize in the cytoplasm or extracellular medium (Figure 3.3b). It was

(a)



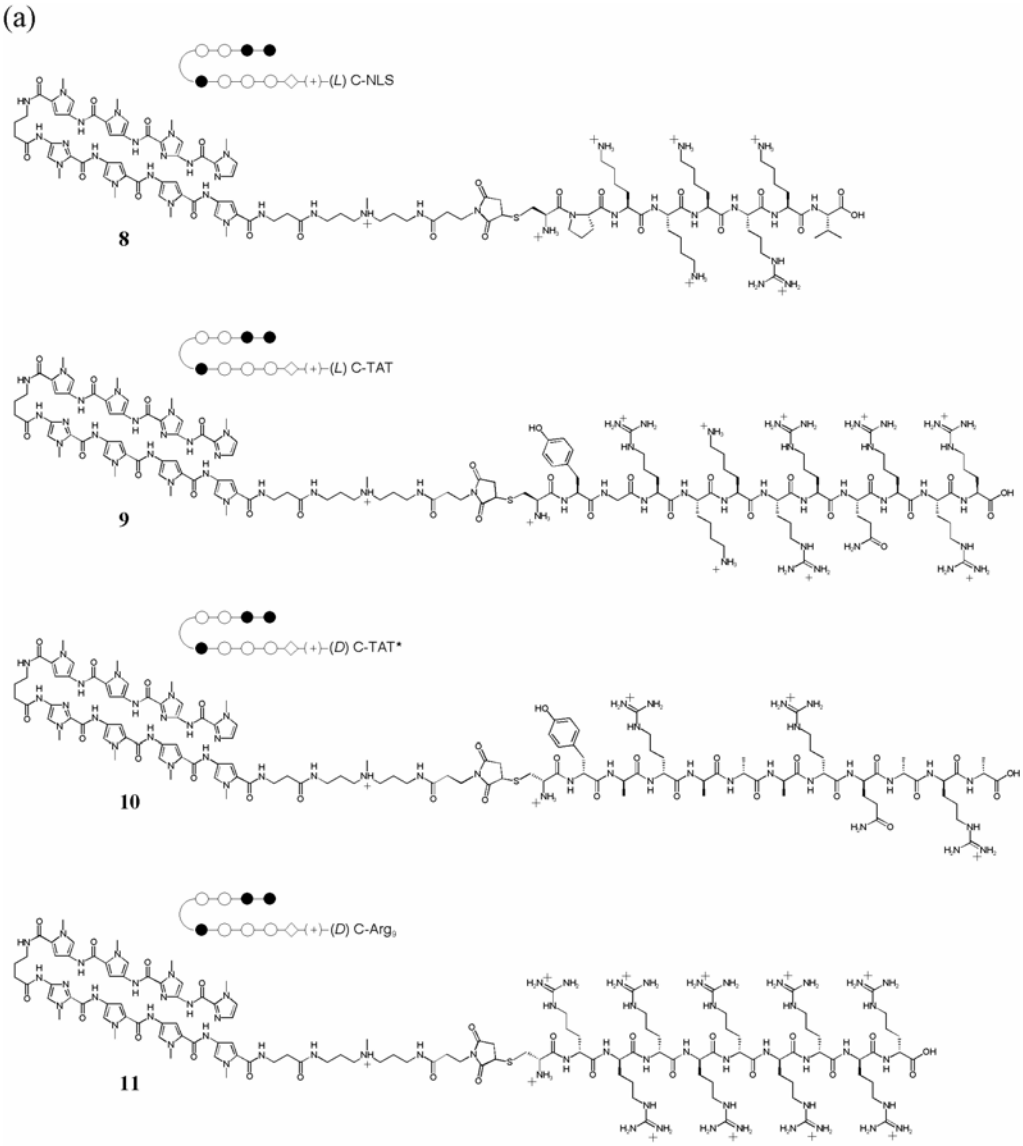
(b)

Conjugate	SKBR-3: human breast cancer	CEM: human cultured T-cells	NB4: human leukemia	293: human kidney fibroblast	Sf9: fall armyworm (insect)	Kc: Drosophila (insect)
4	Cytoplasm	Non-cellular	Non-cellular	Non-cellular	Cytoplasm	Nuclear
5	Cytoplasm	Nuclear	Nuclear	Nuclear	Cytoplasm	Nuclear
6	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Nuclear	Nuclear
7	Nuclear	Nuclear	Nuclear	Nuclear	Nuclear	Nuclear

Figure 3.3 Localization of peptide-polyamide–Bodipy conjugates in live cells as determined by confocal microscopy. (a) Chemical structures and ball-and-stick models of peptide-polyamide-Bodipy conjugates. (b) The designation 'nuclear' indicates observation of fluorescence in the interior of the nucleus. The designation 'cytoplasm' indicates cellular, non-nuclear fluorescence. The designation 'non-cellular' indicates extracellular fluorescence. Cells were imaged directly following 20 h incubation with 5 μM **4**–**7** under normal growth conditions for each cell line.[25]

also found that **5** and **7** tended to be toxic to most cell lines at the concentration used in the assay (5 μM). In order to determine the DNA-binding qualities of polyamide-peptide

(a)



(b)

Conjugate	Match site affinity K_a (M^{-1})	Specificity over coating
8	5×10^8	20-fold
9	$\sim 1 \times 10^9$	None
10	6×10^8	20-fold
11	$\sim 1 \times 10^9$	None

Figure 3.4 DNA-binding characteristics of tail-linked polyamide-peptide conjugates.

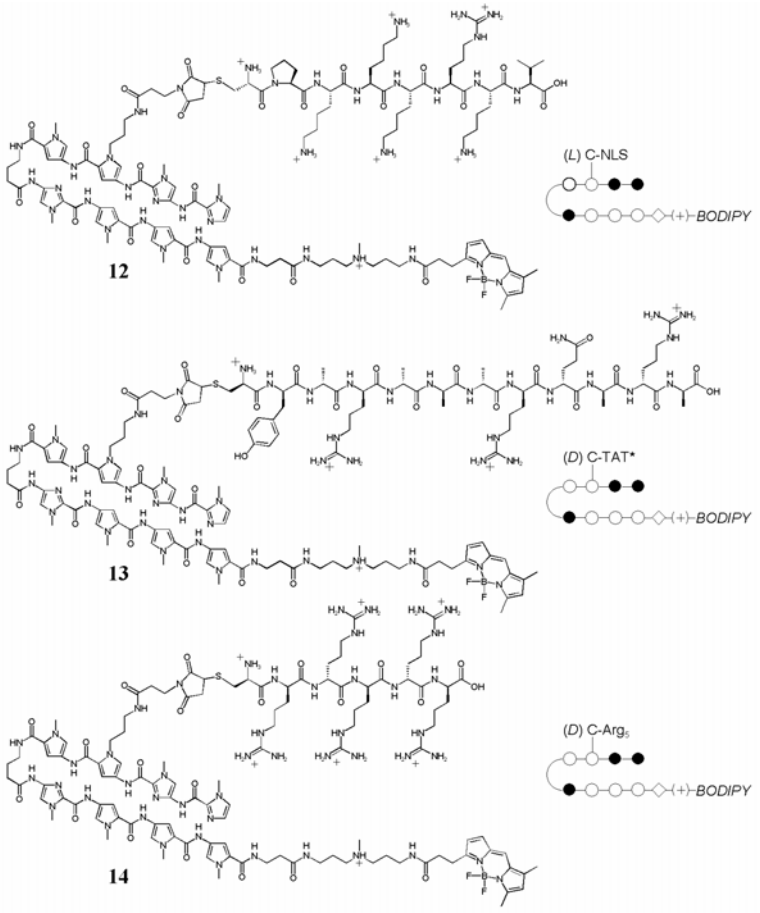
conjugates, compounds **8**-**11** (Figure 3.4a) were synthesized and tested by DNase I footprinting titration.[24] All compounds bound DNA at concentrations at or below 50 nM, with varying degrees of specificity over non-match binding sites. TAT- and (*D*) Arg_9-polyamide conjugates **9** and **11** coated DNA, showing no specificity for any binding site over any other. NLS- and (*D*) TAT*-polyamide conjugates **8** and **10** bound DNA with a specificity for their match sites over all other sites (coating) of 20-fold (Figure 3.4b).[25]

In order to improve the DNA-binding characteristics of polyamide-peptide conjugates, the peptide moiety was moved from the tail to the *N*-methyl position of an internal pyrrole. This arrangement removes the cationic peptides from the DNA minor groove, with the goal of providing compounds with increased affinity and specificity, hopefully retaining or improving their uptake characteristics. Peptide-polyamide-Bodipy conjugates **12**-**14** were synthesized and assayed for their uptake characteristics (Figure 3.5). These compounds had generally poorer uptake characteristics than the tail-linked cognates. Polyamide-peptide conjugates **15**-**17** were then synthesized and assayed for their DNA-binding qualities.

Compounds bearing up to five cationic charges exhibited specificity for binding the DNA match site, at generally higher affinities than the tail-linked counterparts (Figure 3.6). The good uptake characteristics of tail-linked conjugates and the good DNA-binding characteristics of *N*-methyl-linked compounds suggest that molecular shape is an important variable regarding polyamide-peptide conjugates. It was desirable to design a system that incorporated shape characteristics that would promote both good uptake and good DNA affinity and specificity. Such a situation might be achieved by compounds

bearing the peptide moiety on the terminal imidazole residue, rather than an internal residue (Figure 3.7).

(a)

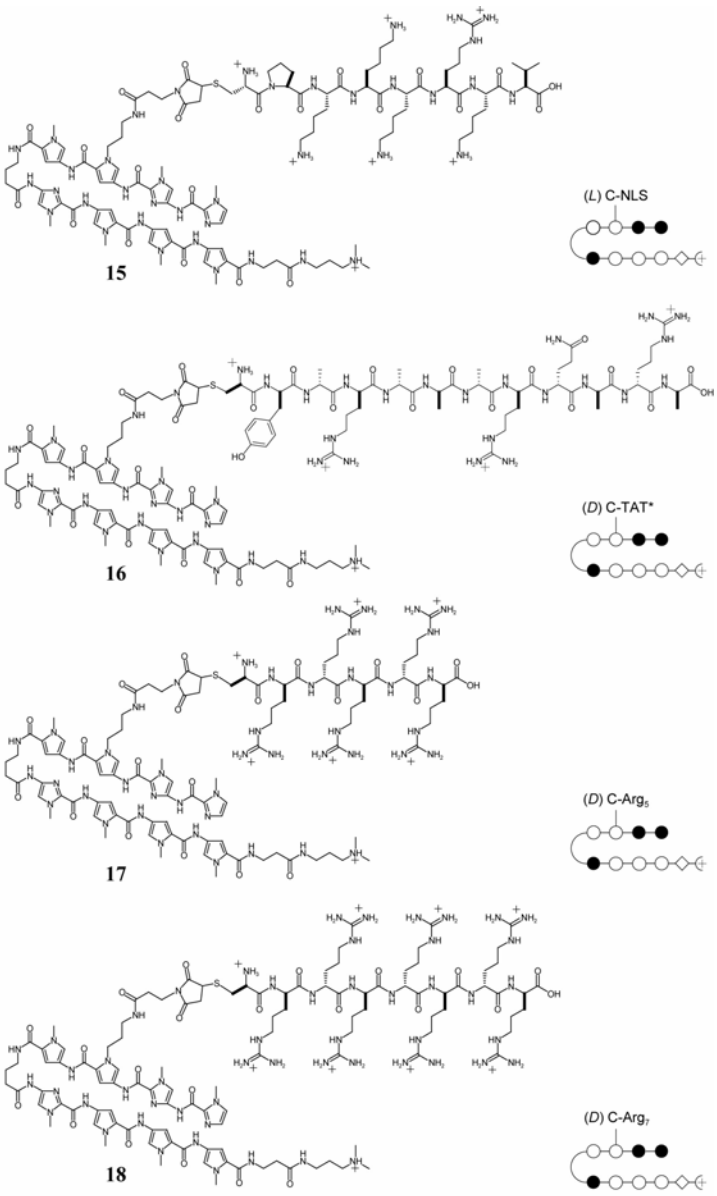


(b)

Conjugate	SKBR-3: human breast cancer	CEM: human cultured T-cells	NB4: human leukemia	293: human kidney fibroblast	Sf9: fall armyworm (insect)	Kc: Drosophila (insect)
12	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
13	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
14	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm

Figure 3.5 Localization of *N*-methyl-linked peptide-polyamide–Bodipy conjugates.[25]

(a)



(b)

Conjugate	Match site affinity K_a (M^{-1})	Specificity over coating
15	1×10^{10}	~100-fold
16	$\sim 3 \times 10^{9}$	~100-fold
17	4×10^{9}	25-fold
18	$\sim 5 \times 10^{8}$	2-5 -fold

Figure 3.6 DNA-binding characteristics of *N*-methyl-linked polyamide-peptide conjugates.[25]

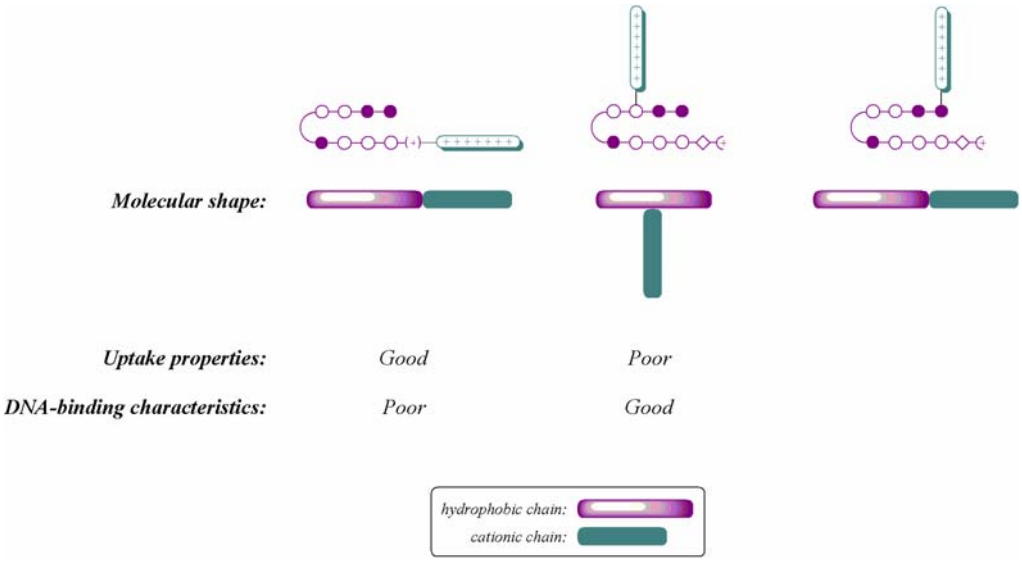


Figure 3.7 Effect of molecular shape on uptake and DNA-binding properties. Attachment of carrier peptides to the terminal imidazole's *N*-methyl position should combine the good uptake properties of tail-linked conjugates and the good DNA-binding properties of *N*-methyl-linked conjugates.

Results

N-terminal imidazole-linked peptide-polyamide-Bodipy conjugates **19**-**24** (Figure 3.8) were synthesized as shown in Figure 3.9. For the conjugation of the peptides to the polyamide, a maleimide-thiol Michael addition reaction was utilized because this reaction proceeds rapidly and selectively in aqueous buffers a physiological conditions. Compounds were added to a panel of fourteen cell lines, consisting of ten human cell lines (PC3, CEM, K562, NB4, MEG, HeLa, Jurkat, SKBR-3, MCF-7, and 786O), two mouse lines (3T3 and MEL), and two insect lines (KC and SF-9). These cell lines represent a wide variety of human cancers, including hard-to-treat varieties such as kidney (786O) and ovarian (HeLa) cancer. Treated cells were imaged by confocal microscopy and assayed for the localization of fluorescence (Figure 3.10).

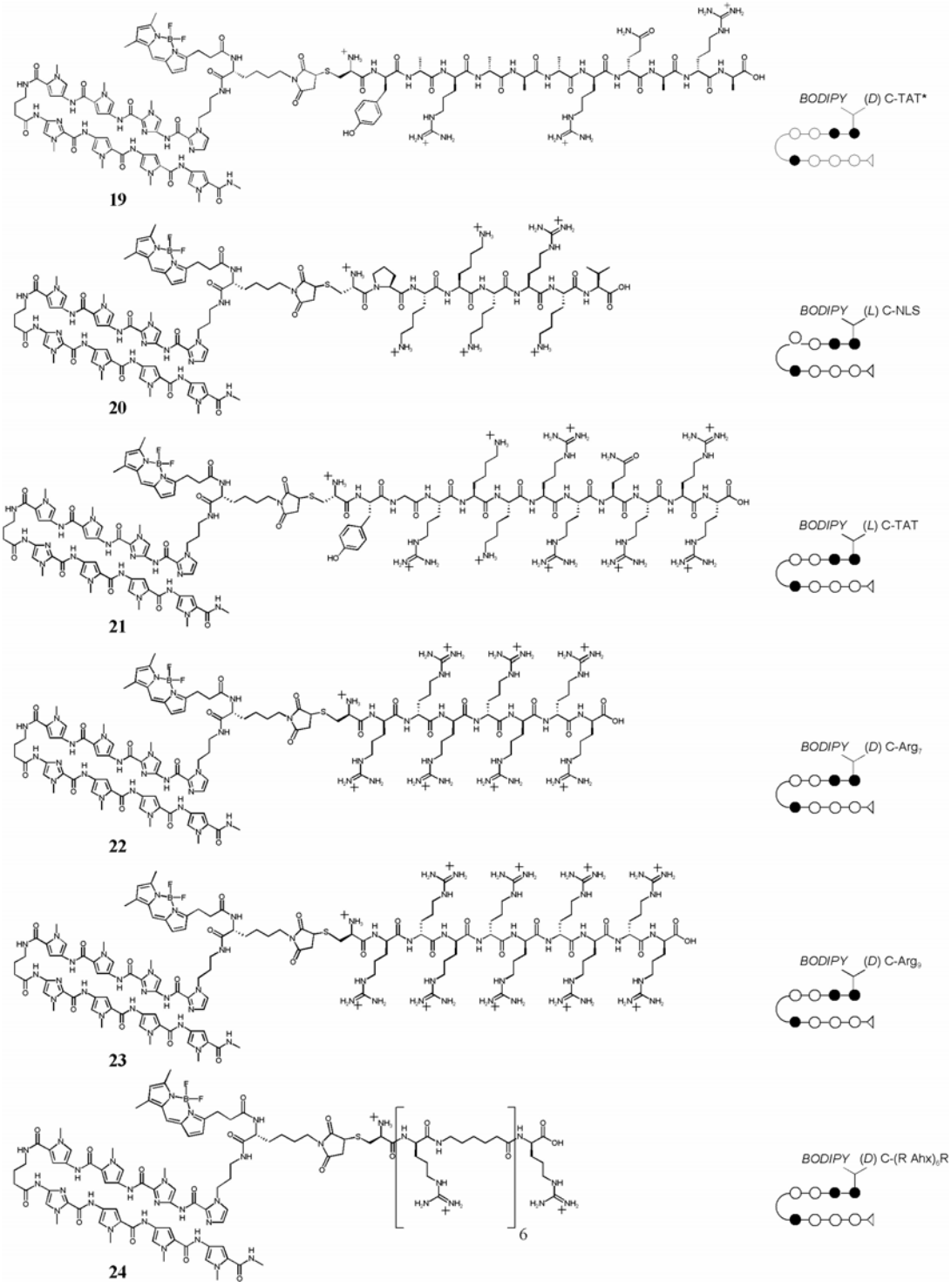


Figure 3.8 N-terminal imidazole-linked peptide-polyamide-Bodipy conjugates for nuclear uptake studies.

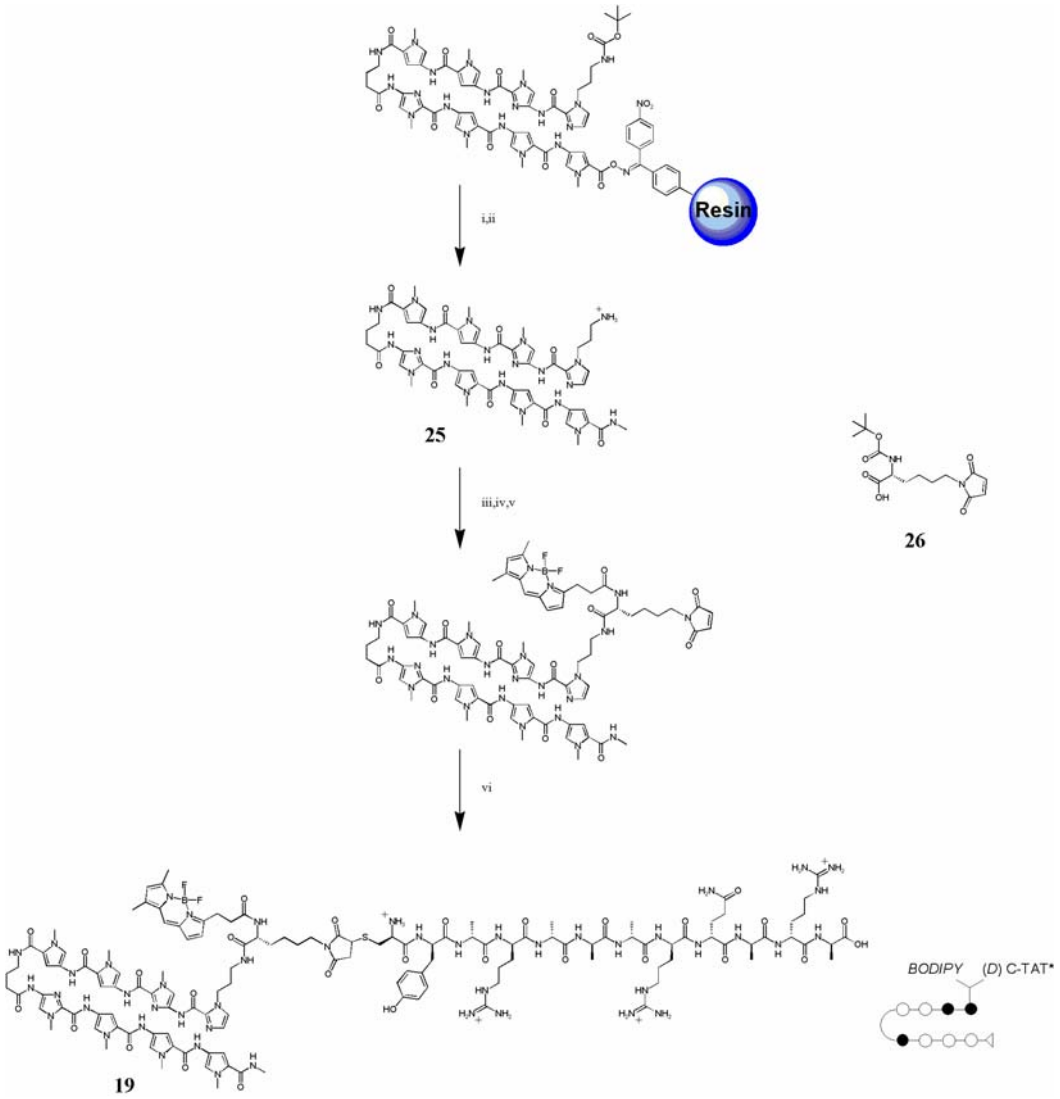


Figure 3.9 Synthesis of a representative compound in the N-terminal-linked series. (i) 1:1 mixture of methylamine (2M in THF):CH_2Cl_2, 6 hrs at 37ºC. (ii) 20% TFA in CH_2Cl_2 (v/v), 30 min at room temperature. (iii) **26**, HOBt, DCC, DIEA, DMF. (iv) 20% TFA in CH_2Cl_2 (v/v), 30 min at room temperature. (v) Bodipy-FL, DCC, HOBt, DIEA, DMF. (vi) TCEP, (*D*) Cys-TAT*, 6M Gn·HCl, pH = 7.0, DMF, 30 min at room temperature.

(a)

Conjugate	PC3	CEM	K562	NB4	MEG	HeLa	Jurkat	SKBR-3	MCF-7	786O	3T3	MEL	Kc	Sf9
19	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt
20	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt
21	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Toxic	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt
22	Cyt	Toxic	Toxic	Toxic	Toxic	Cyt	Toxic	Cyt	Cyt	Cyt	Cyt	Toxic	Cyt	Cyt
23	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Cyt	Toxic	Cyt	Cyt
24	Cyt	Toxic	Cyt	Nuclear	Toxic	Cyt	Toxic	Toxic	Cyt	Toxic	Cyt	Cyt	Cyt	Cyt

(b)

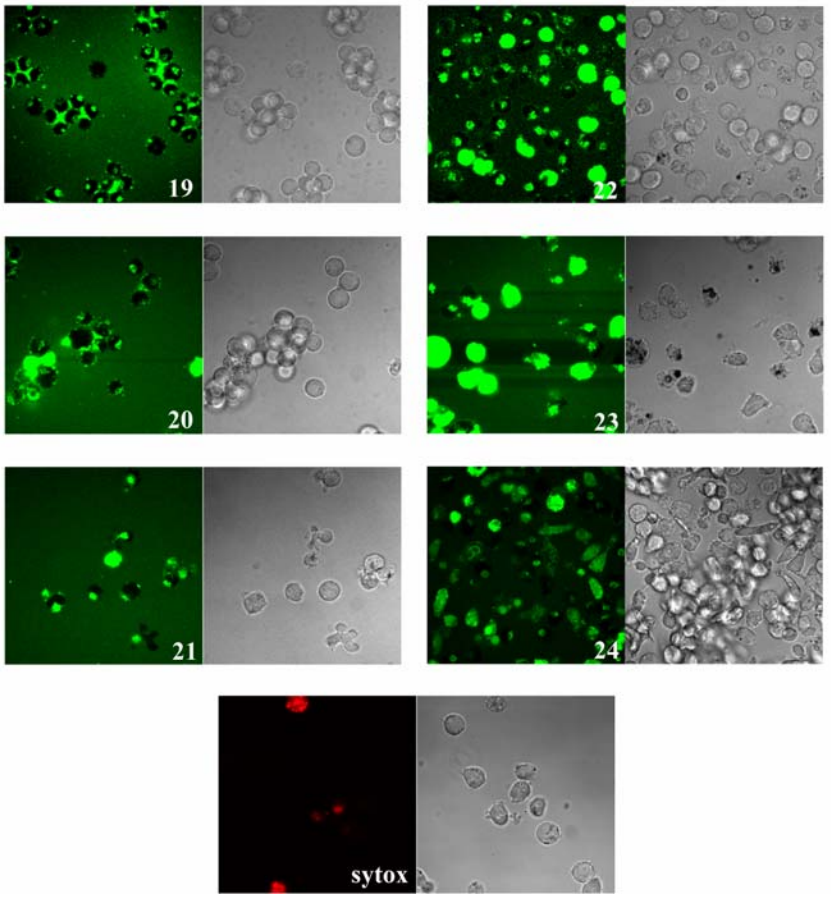


Figure 3.10 Localization of **19**-**24** in live cells as determined by confocal microscopy. (a) The designation 'nuc' indicates observation of fluorescence in the interior of the nucleus. The designation 'cyt' indicates cellular, non-nuclear fluorescence. The designation 'toxic' indicates compound induces toxicity at the concentration studied. Cells were imaged directly following 20 h incubation with 5 μM **19**–**24** under normal growth conditions for each cell-line. (b) Images of **19**-**24** and dead-cell stain Sytox orange in NB4 cells.

In general, compounds **19**-**24** showed poor cellular uptake characteristics similar to those found in internal *N*-methyl-linked conjugates **15**-**18**. Conjugates **19**-**21** were found to be cytoplasmic (or in the case of Jurkat, toxic) in all cell lines studied. Polyarginine conjugates **22**-**24** showed either cytoplasmic staining or toxicity, with Arg_9 conjugate **23** being the most toxic compound in each human cell line studied. The sole entry showing somewhat promising localization was compound **24** in NB4 cells. The $(R\text{-}Ahx)_6R$ peptide was developed by Wender and co-workers as a superior carrier peptide in jurkat cells.[26] In this assay it was generally too toxic to be useful as a carrier at the concentrations studied.

Though the uptake characteristics of the N-terminal imidazole-linked peptide conjugates were not promising, the DNA-binding characteristics of this system were nonetheless interesting to determine. To that end, compounds **27**-**32** (Figure 3.11) were synthesized and assayed by DNase I footprinting on the plasmid pDEH9, which bears a match 5'-TGGTCA-3' site and discrete mismatch binding sites 5'-TGGCCA-3' and 5'-TGGGCA-3' for the polyamide unit used in this study (Figure 3.12a). Analysis of the footprinting gels (Figure 3.12b) shows that the DNA-binding characteristics of N-terminal-linked conjugates are intermediate between tail linkage and internal *N*-methyl linkage. (*D*) TAT*- and NLS-conjugates **27** and **28** show the best specificity for their DNA match site over nonspecific binding (coating). Surprisingly, the $(R\text{-}Ahx)_6R$-conjugate **32** shows some specificity for its match site over coating (10-20 fold) even though the R_7-conjugate **30**, bearing the same overall molecular charge, shows negligible specificity over coating. The poor DNA-binding qualities of the polyarginine conjugates, coupled with their toxicity to cells, makes them unsuitable for conjugation to polyamides

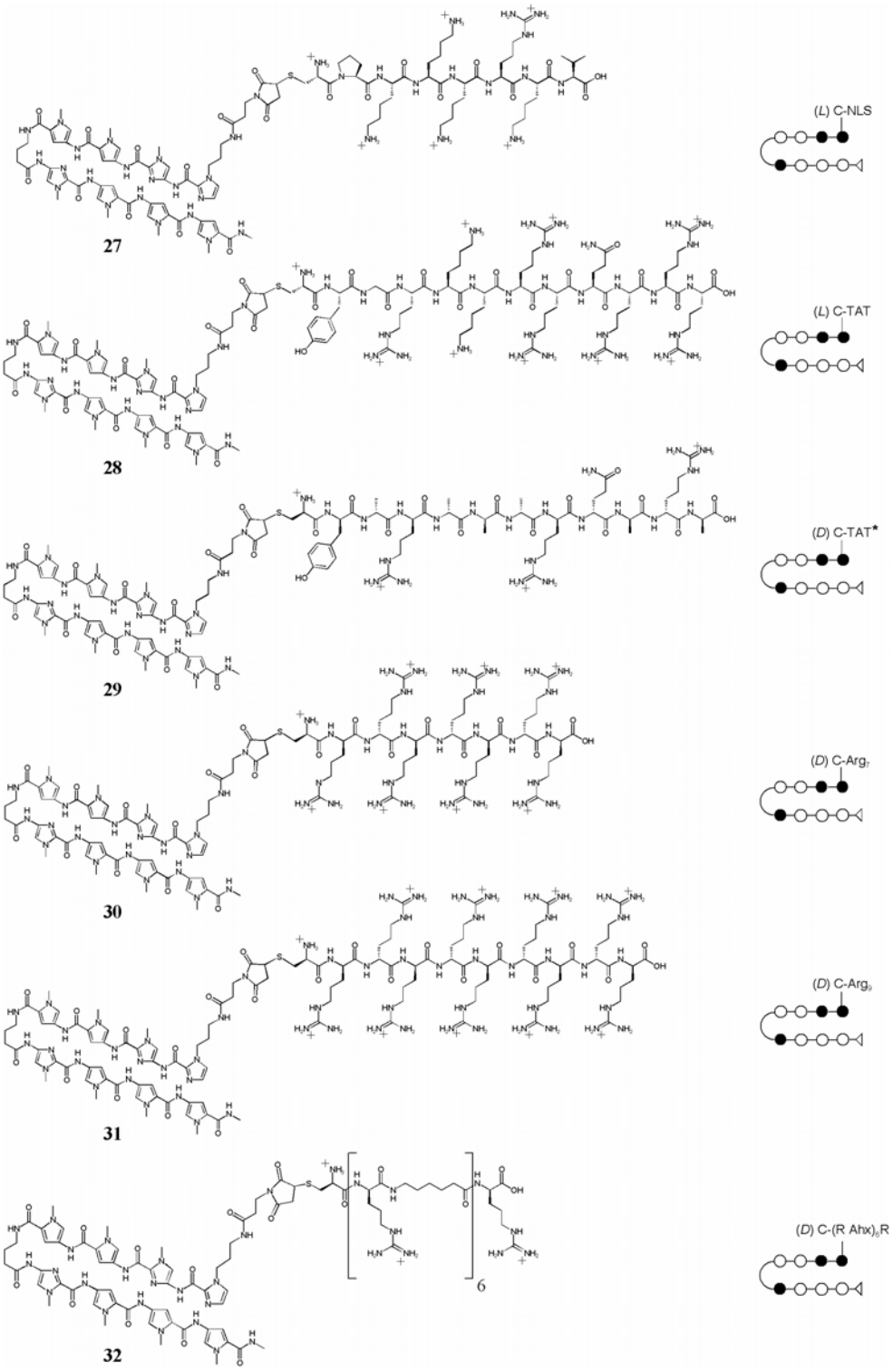


Figure 3.11 N-terminal imidazole-linked polyamide-peptide conjugates for footprinting studies.

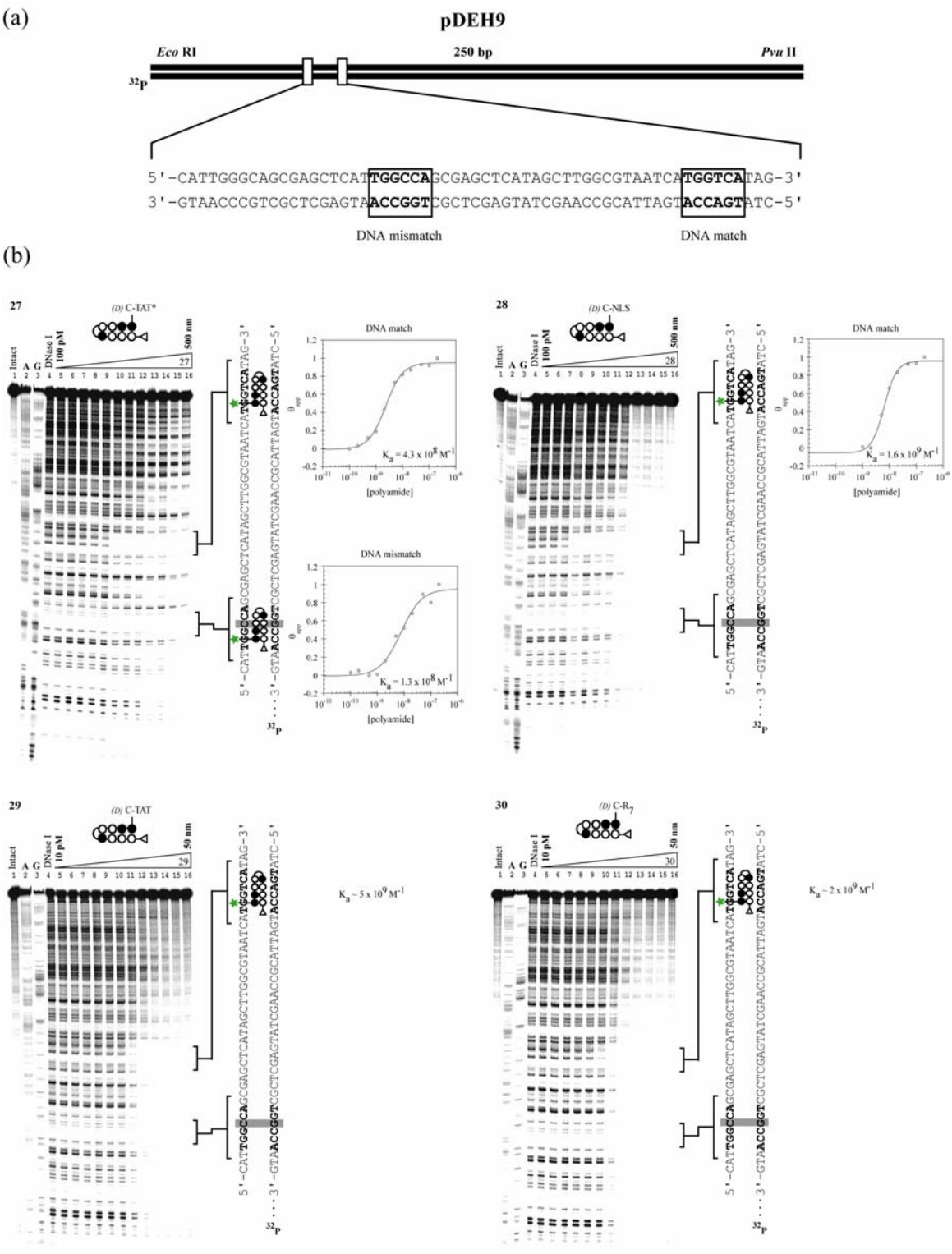
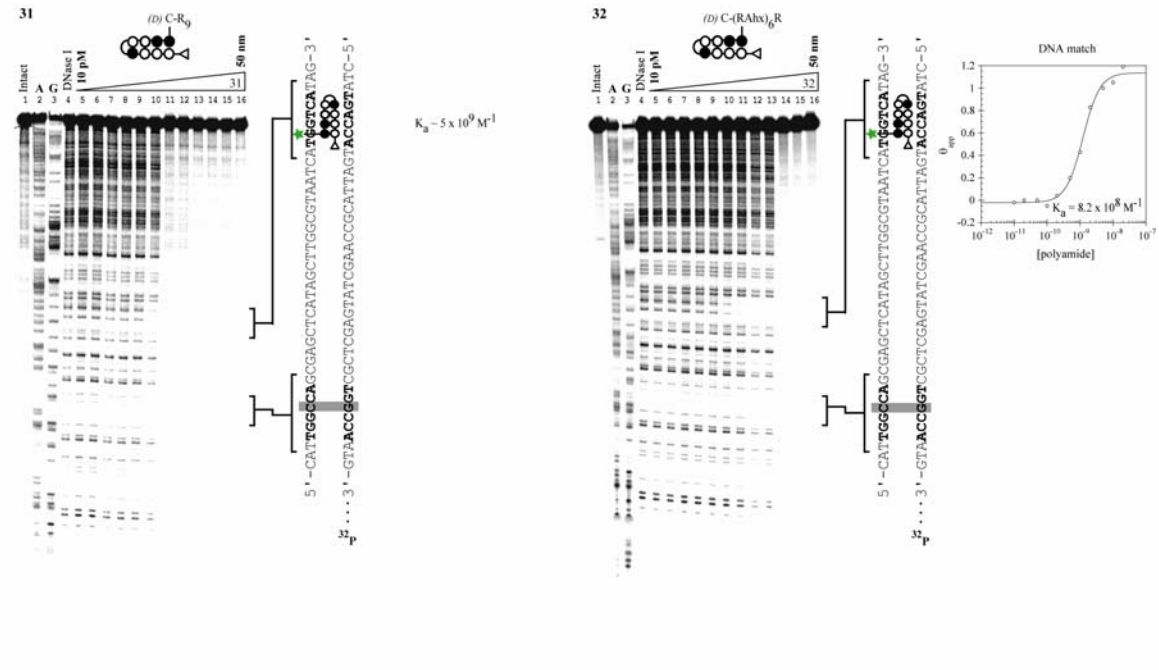


Figure 3.12 DNA-binding characteristics of N-terminal imidazole-linked polyamide-peptide conjugates. (a) Plasmid pDEH9 illustrating match and mismatch sites for the polyamide used in this study. (b) DNase I footprinting gels for **27**-**32**, showing isotherms where measurable.



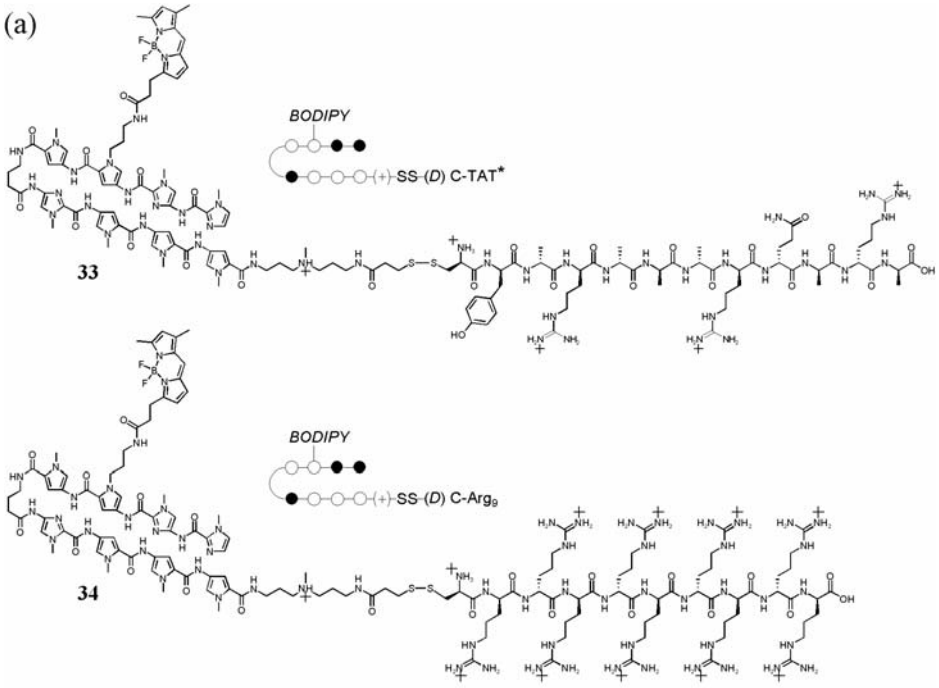
(c)

Conjugate	Match site affinity K_a (M^{-1})	Specificity over coating	Conjugate	Match site affinity K_a (M^{-1})	Specificity over coating
8 + –(*L*) C-NLS	5×10^8	20-fold			
9 + –(*L*) C-TAT	~1×10^9	None	**27** (*L*) C-NLS	2×10^9	25-fold
10 (+)–(*D*) C-TAT*	6×10^8	20-fold	**28** (*L*) C-TAT	~5×10^9	None
11 (+)–(*D*) C-Arg_9	~1×10^9	None	**29** (*D*) C-TAT*	4×10^8	~100-fold
15 (*L*) C-NLS	1×10^{10}	~100-fold	**30** (*D*) C-Arg_7	~3×10^9	2-5 -fold
16 (*D*) C-TAT*	~3×10^9	~100-fold	**31** (*D*) C-Arg_9	~5×10^9	None
17 (*D*) C-Arg_5	4×10^9	25-fold	**32** (*D*) C-(R Ahx)$_6$R	8×10^8	10-20 -fold
18 (*D*) C-Arg_7	~5×10^8	2-5 -fold			

Figure 3.12 DNA-binding characteristics of N-terminal imidazole-linked polyamide-peptide conjugates. (c) Comparison of DNA-binding characteristics of carrier peptide-polyamide conjugates in three morphologies. In general **27**-**32** possess intermediate binding characteristics between tail-linked **8**-**11** and internally-linked **15**-**18**.

for use as DNA-binding ligands in living systems.

One possible way to overcome the poor DNA-binding characteristics of carrier peptide-polyamide conjugates is by employing a pro-drug approach, attaching a carrier moiety to a polyamide via a linking domain that will be cleaved upon entering the cellular environment. One such linking strategy is the disulfide bond, which is cleaved upon entering the reductive cytoplasmic environment to the constituent thiols.[8] This strategy was implemented by the synthesis of compounds **33** and **34**, containing a Bodipy-polyamide moiety linked to either (*D*) TAT* or Arg_9 through a disulfide bond



(b)

Conjugate	PC3	CEM	NB4	MEG	HeLa	Jurkat	MCF-7	786O	MEL
33	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt
34	Cyt	Cyt	Toxic	Toxic	Cyt	Cyt	Cyt	Cyt	Toxic

Figure 3.13 Disulfide-linked polyamide-peptide prodrugs. (a) Chemical structures and ball-and-stick models of **33** and **34**. (b) Uptake profile of disulfide conjugates in nine mammalian cell lines.

(Figure 3.13a). These conjugates were synthesized according to the scheme outlined in Figure 3.14. Upon exposure to a panel of mammalian cell lines, **33** and **34** were assayed for cellular uptake by confocal microscopy (Figure 3.13b). Unfortunately, neither conjugate was successful in staining the nucleus of any cell line. Arg_9 remained a toxic constituent in some cell lines. In general, carrier peptide conjugates do not seem to be a fruitful path forward in promoting uptake of polyamides. It is likely that small-molecule conjugates will be a more successful approach to the problem of utilizing polyamides in living systems.

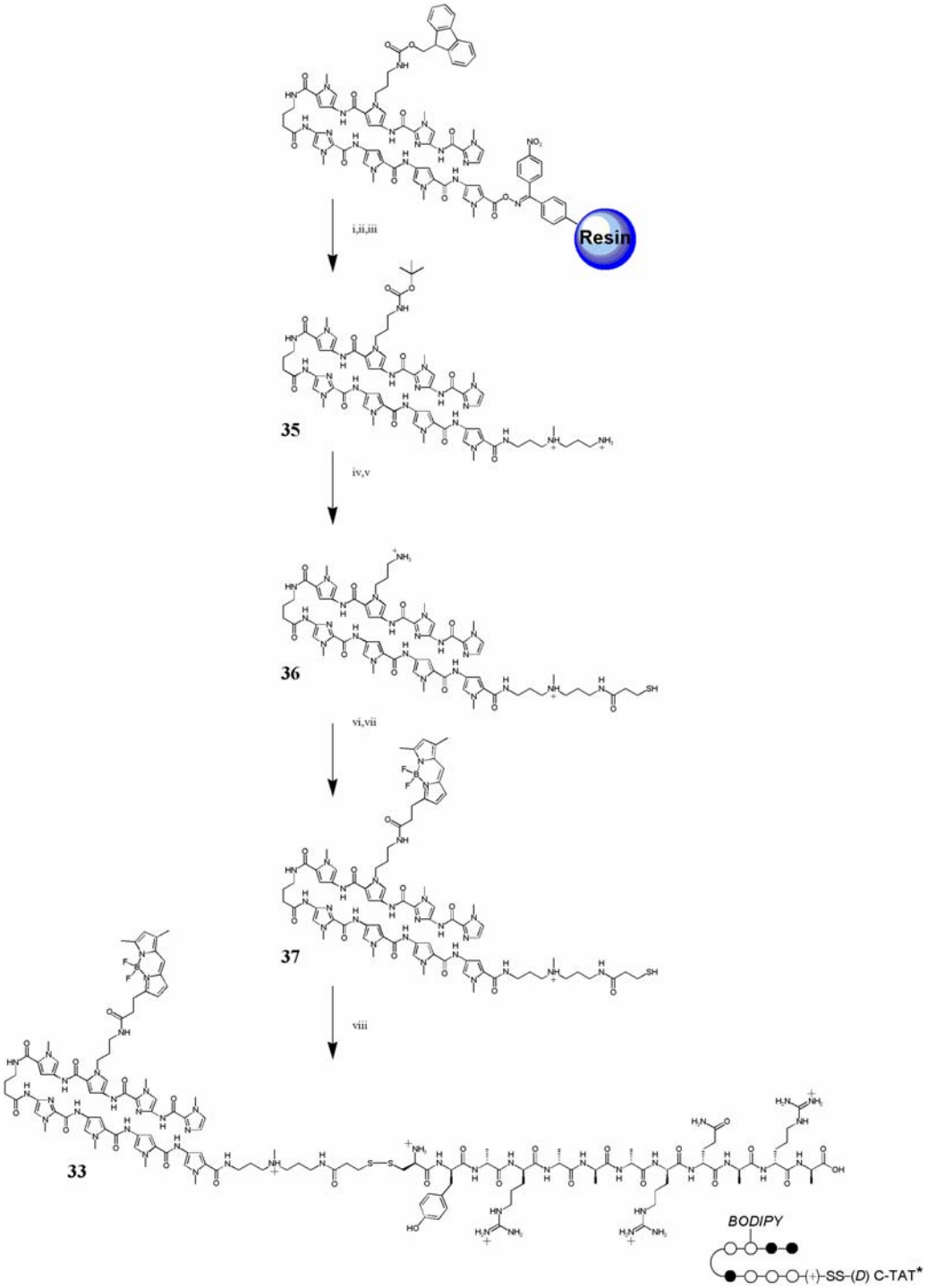


Figure 3.14 Synthesis of disulfide conjugates. (i) 20% piperidine/DMF (v/v), 30 min at room temperature. (ii) Boc_2O, DIEA, DMF. (iii) 3,3'-diamino-*N*-methyldipropylamine, 6 hr at 37ºC. (iv) *S*-trityl-3-mercaptopropionic acid, HOBt, DCC, DIEA, DMF. (v) 50% TFA in CH_2Cl_2 (v/v), 1 hr at room temperature. (vi) Bodipy-FL, HOBt, DCC, DIEA, DMF. (vii) 3-dimethylaminopropylamine, 2 hr at 37ºC. (viii), 2-Aldrithiol, (*D*) Cys-TAT*, DMF, 4 hrs at room temperature.