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Combining Aminocyanine Dyes with Polyamide Dendrons: A Promising Strategy for Imaging in the Near-Infrared Region

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Abstract: Cyanine dyes are known for their fluorescence in the near-IR (NIR) region, which is desirable for biological applications. We report the synthesis of a series of aminocyanine dyes containing terminal functional groups such as acid, azide, and cyclooctyne groups for further functionalization through, for example, click chemistry. These aminocyanine dyes can be attached to polyfunctional dendrons by copper-catalyzed azide alkyne cycloaddition (CuAAC), strain-promoted azide alkyne cycloaddition (SPAAC), peptide coupling, or direct $S_{NR}1$ reactions. The resulting dendron–dye conjugates were obtained in high yields and displayed high chemical stability and photostability. The optical properties of the new compounds were studied by UV/Vis and fluorescence spectroscopy. All compounds show large Stokes shifts and strong fluorescence in the NIR region with high quantum yields, which are optimal properties for in vivo optical imaging.

Keywords: CuAAC · cycloaddition · dendrimers · dendrons · dyes/pigments · imaging agent · SPAAC

Introduction

In life science, the search for new methods of early detection of diseases has stimulated researchers to target the development of efficient imaging agents.[1–5] Different imaging modalities present different strengths and weaknesses. For example, magnetic resonance imaging (MRI) has a very high spatial resolution but its sensitivity of around 10^{-3}–10^{-5} M can be limiting.[4] In some particular cases, positron emission tomography (PET) is more appropriate due to its high sensitivity.[1,6,7] Among the different imaging modalities, optical imaging is the one that presents less safety concerns, because organic fluorescent dyes often display good biocompatibility, a significant advantage over radioactive markers, which are frequently toxic.[2] Thus, intensive research efforts have been devoted to the design and synthesis of new water-soluble organic fluorophoric systems.[3,8] The optimal wavelength range for in vivo fluorescence excitation and emission is determined by the optical properties of the tissue of interest. Hemoglobin has a strong absorption at wavelengths lower than 600 nm and significant background fluorescence from endogenous biomolecules can be detected up to 680 nm.[8] Near infrared (NIR) light can overcome these limitations by penetrating more deeply into tissue, because light scattering decreases with increasing wavelength. Thus, organic fluorophores with absorption and emission properties in the NIR region are ideal for in vivo optical imaging.[8]

Among the organic near-infrared dyes, cyanine-based compounds are of particular interest. Their basic structure includes two aromatic or heterocyclic rings linked by a polymethine chain with conjugated carbon–carbon bonds.[9,10] These compounds have found their use in day-to-day applications in the medical field. For example, indocyanine green (ICG) (Scheme 1) is a Food and Drug Administration (FDA)-approved cyanine dye that is currently used for imaging in humans.[8] It is considered a safe imaging agent and has been used in tens of thousands of patients with a reported side-effect rate below 0.15%, an extremely favorable index when compared to other imaging agents.[8,11–13] This impressive biocompatibility has initiated a search for cyanine dye derivatives containing functional handles available for further functionalization in order to attach these dyes to peptides, targeting groups and/or drug carriers.[10] Unfortunately, the synthesis of ICG derivatives is often difficult and low yielding mainly due to low solubility and chemical stability.[9,10] One strategy to overcome these shortcomings has been introduced by Patonay and co-workers who showed that the insertion of a cyclohexenyl ring into the polymethine chain increases solubility and photostability and provides an additional site for further functionalization (Scheme 1).[14]

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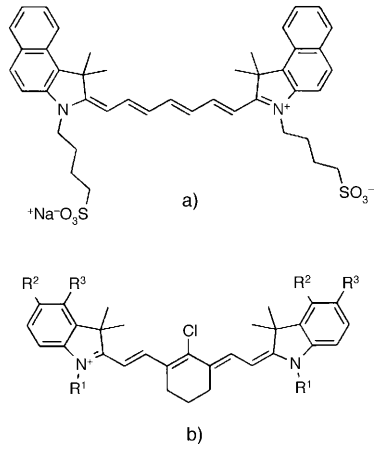


Scheme 1. a) Indocyanine green (ICG) and b) cyanine dye containing a cyclohexenyl ring on the polymethine chain.

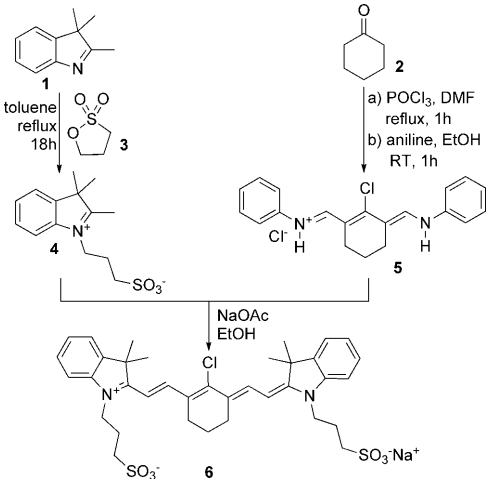
Alcohol[15–18] or thiol[19–22] groups are often employed to replace the chlorine atom at the central ring. The resulting ethers or thioethers unfortunately present relatively low chemical stability limiting their use as fluorescent probes in vivo.[23,24] The Achilefu group has reported the use of a cross-coupling reaction as an alternative reaction to functionalize cyclohexene-containing cyanine dyes resulting in cyanine derivatives with C–C bonds at the central ring.[23] These dyes are more stable than their ether or thioether analogues. However, their optical properties are not optimal due to the low stokes shift (<30 nm).[23] Gao and co-workers have shown that the replacement of the chlorine atom with an amine group results in the formation of aminocyanine dyes with high quantum yields and large Stokes shifts (>140 nm).[24] These materials have been explored as fluorescent probes for metal ions, however, reports about their use as imaging agents are still scarce.[25–29]

Our research group has been developing synthetic strategies for the synthesis of new drug delivery systems based on well-defined multifunctional dendrons and dendrimers.[30–33] We were very intrigued by the above-described aminocyanine dyes as potential imaging agents for our multifunctional dendrimer-based delivery system for applications in theranostics.[34–43] Orthogonality between functional groups is crucial to the synthesis and functionalization of such complex structures,[44] thus, our attention was focused on the derivatization of the NIR dyes with functional groups suitable for orthogonal chemistry. In this contribution we present an extensive study on functionalization strategies for chlorine-containing cyanine dyes with a library of amines containing different functional groups and evaluated the chemical stability and optical properties of the resulting materials. The dyes were then attached to either one terminal branch or the focal point of polyfunctional dendrons by using copper-catalyzed alkyne azide cycloaddition (CuAAC), the most popular "click" reaction,[45–50] strain-promoted alkyne azide cycloaddition (SPAAC),[33,51–53] peptide coupling, or direct radical nucleophilic substitution ($S_{RN}1$) reactions. The dendrons employed in this work are polyamide-based materials and follow the 1→(2+1) connectivity pioneered by Newkome et al.[54] All aminocyanine dyes and dendron–dye conjugates were characterized by using NMR spectroscopy, mass spectrometry, and elemental analysis, and their optical properties were investigated by UV/Vis and fluorescence spectroscopy. The new compounds show large Stokes shifts and strong fluorescence in the NIR region with high quantum yields, which are optimal properties for in vivo imaging. Furthermore, the dendrons possess terminal groups available for further functionalization with targeting groups and/or drugs, making these materials suitable for theranostics.

Results and Discussion

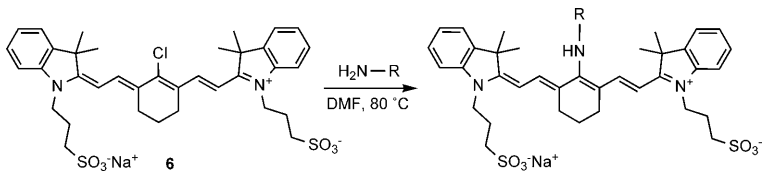
Because our main goal is the use of NIR dyes as imaging agents for biological applications, we investigated cyanine dyes that contain two sulfonate groups at the end of the propyl chains on the nitrogen of the indolenine ring, which are known to increase water solubility.[16] Furthermore, the negative charges on the sulfonate groups should enhance the biocompatibility of the dye–dendron conjugates because negatively charged dendrimers are less toxic than their positively charged analogues.[35,36]

Synthesis of the aminocyanine dyes: The chlorocyanine dye was synthesized in three steps from 2,3,3-trimethylindolenine (**1**), cyclohexanone (**2**), and 1,3-propane sultone (**3**) in close analogy to the literature (Scheme 2).[15,16] The hepta-



Scheme 2. Synthesis of the NIR chlorocyanine dye **6**.

methine chain of **6** was assembled from the aldol-like condensation of the indolinium salt **4** and the iminium salt **5** (Scheme 2). Dye **6** was obtained as a deep green solid in 70% overall yield and characterized by mass spectrometry showing its molecular ion peak $[M-Na]^-$ at *m/z* 697.4 (*m/z* calcd for $C_{36}H_{42}ClN_2O_6S_2$: 697.2).



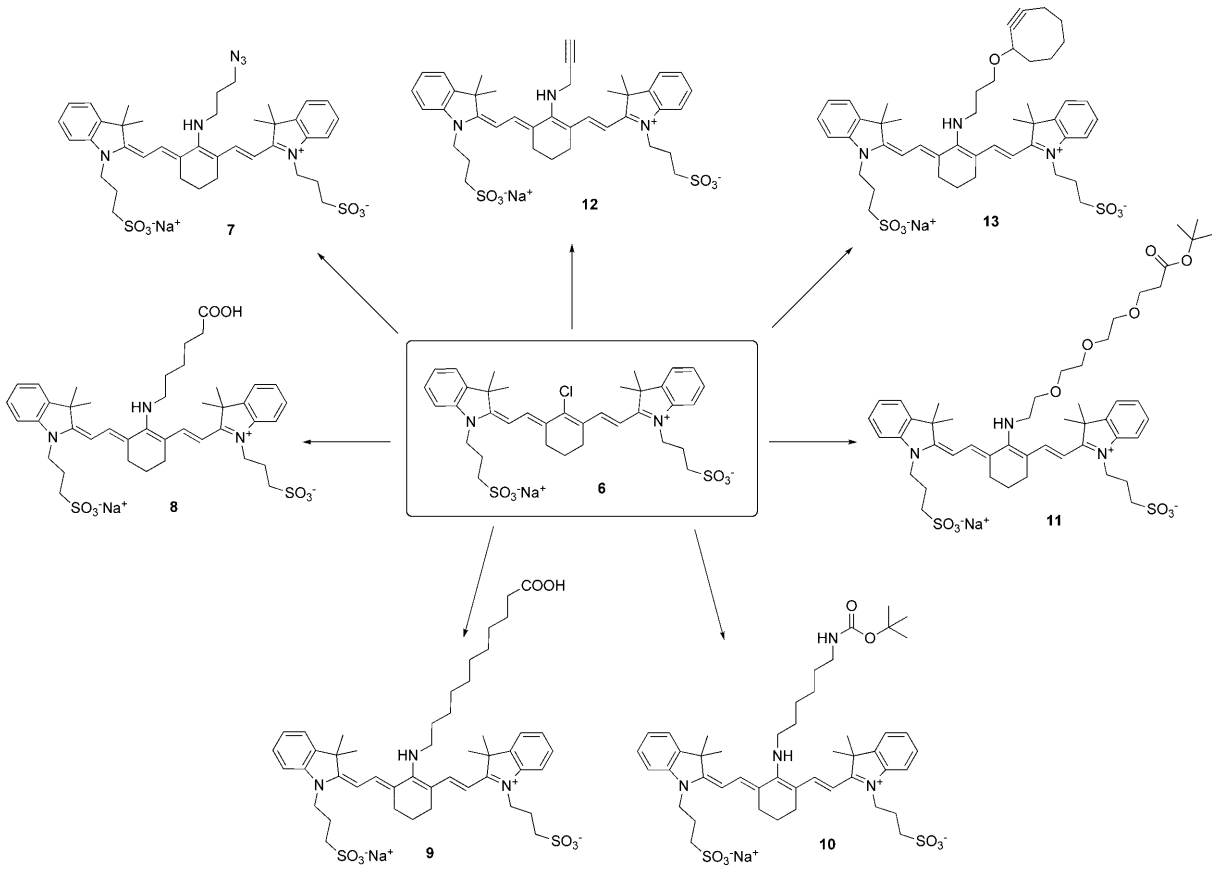
Scheme 3. General $S_{RN}1$ reaction of chlorocyanine dye **6** with various amines.

The reaction of **6** with amines was carried out in dimethylformamide (DMF) at 80 °C.[24] In most cases, the substitution of the chlorine atom by the amine group (Scheme 3) was observed easily by a color change of the initial deep green solution to deep blue. Because our target was the conjugation of the dyes to dendritic molecules by using high yielding reactions, such as peptide coupling, CuAAC, and SPAAC, we focused on the funtionalization of **6** with acid, amine, azide, and alkyne terminal groups.

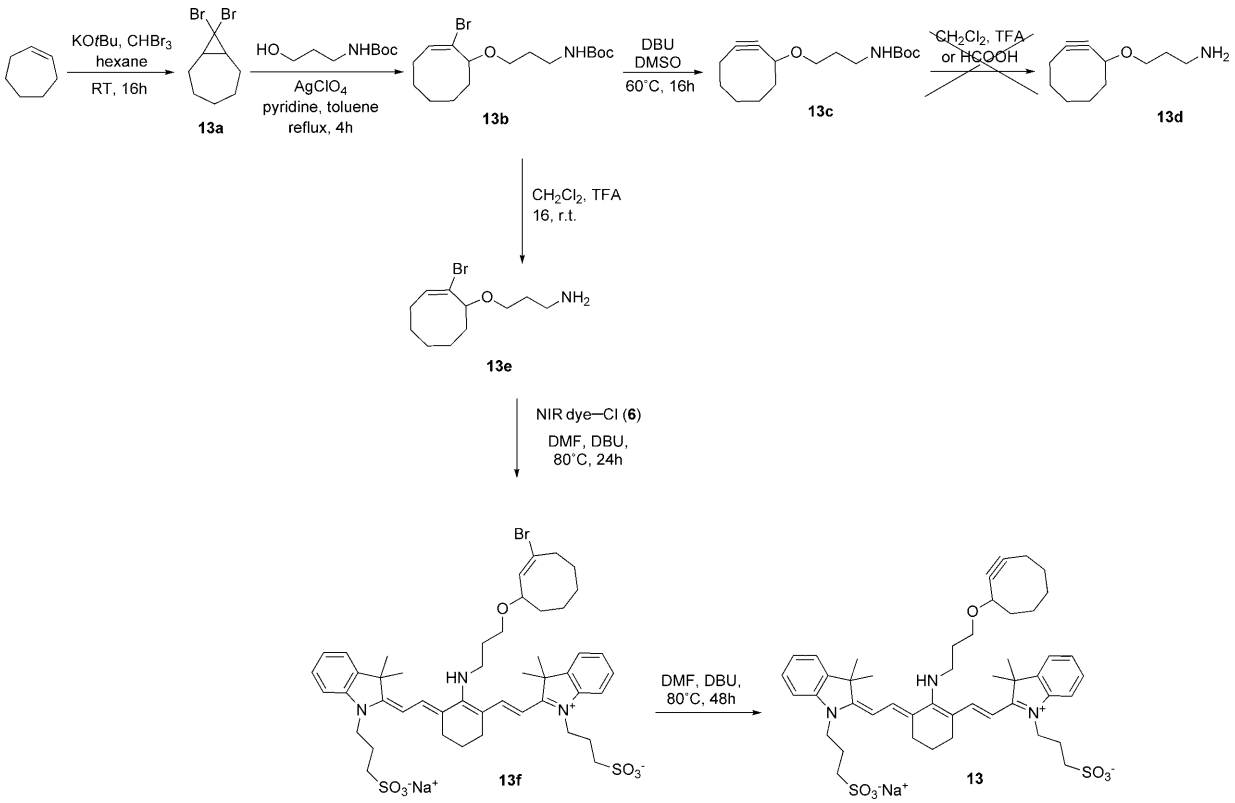
The $S_{RN}1$ reactions of **6** with 3-aminopropyl azide, 6-aminohexanoic acid, 11-aminoundecanoic acid, *N*-Boc-1,6-hexanediamine (Boc = *tert*-butoxycarbonyl), and *tert*-butyl 12-amino-4,7,10-trioxadodecanoate were carried out successfully yielding dyes **7**–**11** (Scheme 4). After precipitation with ethyl ether, these dyes were obtained as deep blue solids. Final purification was performed by dialysis against water and the materials were characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (see the Supporting Information).

To obtain a NIR dye with an alkyne group, a $S_{RN}1$ reaction between **6** and propargyl amine was carried out. After heating at 80 °C for 2 h, the reaction mixture was analyzed by mass spectrometry, which showed the molecular ion peak $[M-Na]^-$ of **12** at *m/z* 716.4 (*m/z* calcd for $C_{39}H_{46}N_3O_6S_2$: 716.3). However, even after several attempts, compound **12** could not be isolated probably due to low chemical stability. We suggest that the close proximity of the alkyne group to the cyanine π system could be the cause of the low stability.

A synthetic strategy was designed to obtain the dye–cyclooctyne conjugate with an amine-containing cyclooctyne ring (**13d**) as the key intermediate (Scheme 5). Bicyclic compound **13a** (8,8-dibromobicyclo[5.1.0]octane) was synthesized according to the procedure described by Skattebol and Solomon for the synthesis of 9,9-dibromo[6.1.0]nonane.[55,56] Silver perchlorate was then used for the electrocyclic ring-opening to the *trans*-allylic cation, which was captured with 3-(Boc-amino)-1-propanol affording the bromo-*trans*-cyclo-



Scheme 4. Functionalization of the chlorocyanine dye **6** with a library of amines.



Scheme 5. Synthesis of dye–cyclooctyne **13**. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TFA = trifluoroacetic acid.

octene **13b**. Cyclooctyne **13c** was obtained by elimination of bromide in DMSO at 60 °C, by using DBU as the base. However, the resulting cyclooctyne ring was not stable under acidic conditions (formic acid or TFA) used for the deprotection of the Boc group. Thus, we removed the Boc protecting group and attached the resulting amine to **6** prior to the elimination reaction affording **13**. The reaction of amine **13e** with **6** and the elimination were carried out by using a one-pot procedure. Amine **13e** and **6** were heated at 80 °C in DMF together with two equivalents of DBU. After 24 h, a mass spectrum of the reaction showed the molecular ion peak $[M-Na]^-$ of **13f** at *m/z* 922.4 (*m/z* calcd for $C_{47}H_{61}BrN_3O_7S_2$: 922.3) suggesting that the substitution of the chlorine atom by the amine group was complete. However, no evidence for the elimination reaction was visible. Ten more equivalents of DBU were added and the solution was heated at 80 °C for another 48 h. After this time, the mass spectrum of the solution showed the molecular ion peak $M-Na]^-$ of **13** at *m/z* 842.6 (*m/z* calcd for $C_{47}H_{60}N_3O_7S_2$: 842.4) (see the Supporting Information for the mass spectra).

Optical properties of the aminocyanine dyes: All aminocyanine dyes **7**–**13** are deep blue in solution and the solid state. The general features of their absorption spectra are: 1) a strong blue shift when compared to **6**, from 780 nm to 595–645 nm, 2) a large stokes shift (>105 nm), and 3) no mirror image relationship between the absorption and fluorescence spectra (Table 1). These features are in agreement with previous reports on aminocyanine dyes and result from an intramolecular charge transfer (ICT).[24,28] The maximum absorption of the dyes is highly solvent dependent (Table 1). However, contrary to the reported aminocyanine dyes with alkyl or phenyl termini, the solvatochromism of the new

Table 1. Optical properties of the aminocyanine dyes.

Dye	Absorption[a]					Emission[b]	
	PBS λ [nm] (ε)	DMSO λ [nm] (ε)	DMF λ [nm] (ε)	EtOH λ [nm] (ε)	MeOH λ [nm] (ε)	λ [nm]	ϕ_F[c]
7	622 (1500)	632 (2400)	624 (1400)	636 (3200)	637 (4000)	755	0.13
8	611 (12900)	634 (13100)	617 (11600)	629 (18300)	628 (21500)	758	0.10
9	607 (3400)	634 (4000)	614 (3400)	629 (5600)	623 (6400)	752	0.13
10	616 (30500)	635 (31300)	620 (28000)	635 (36200)	628 (39800)	752	0.11
11	633 (33600)	645 (31900)	635 (25500)	648 (40400)	648 (44000)	754	0.16
13	595 (2100)	636 (4100)	616 (2600)	627 (6300)	613 (7600)	753	0.11

[a] The absorption spectra were recorded at 25 °C and ε is expressed in $M^{-1}cm^{-1}$ at the maximum of the highest peak. PBS = phosphate buffer aqueous solution. [b] Excitation wavelength was 632 nm for all compounds. [c] Relative fluorescence quantum yields (ϕ_F) were determined in methanol by using the known quantum yield of ICG as a reference ($\phi_F = 0.078$ in methanol).[11]

dyes does not follow a specific trend based on solvent polarity. For dyes **8**–**10**, which possess an alkyl linker between the dye and the functional group, the value of absorption (λ_{max}) follows the trend DMSO > EtOH > MeOH > DMF > PBS, whereas for dye **11**, in which the linker is a polyethyleneglycol chain, the absorption λ_{max} follows the trend MeOH = EtOH > DMSO > DMF > PBS (Table 1, Figure 1). When comparing the absorption spectra of the aminocyanine dyes in the same solvent, their λ_{max} also show variations. These results suggest that the terminal functional group, as well as the nature and length of the linker have a strong influence on dye–solvent interactions.

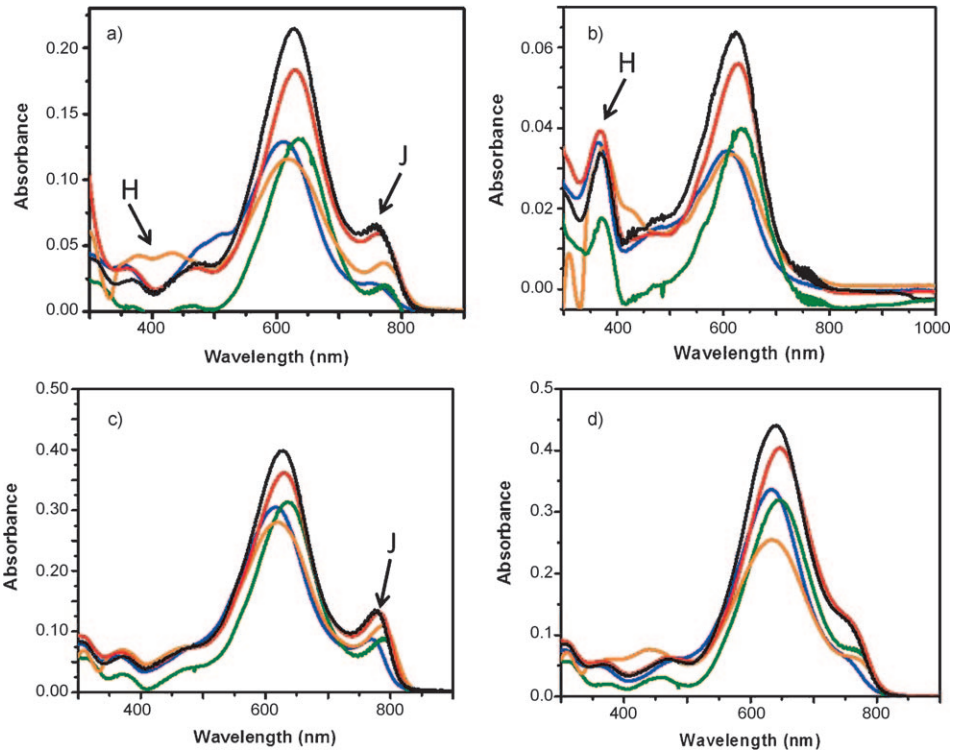


Figure 1. Absorption spectra (concentrations of 1×10^{-5} M at 25 °C) of a) dye–$(CH_2)_5$–COOH (**8**), b) dye–$(CH_2)_{10}$–COOH (**9**), c) dye–$(CH_2)_6$–NHBoc (**10**), and d) dye–PEG–COO*t*Bu (**11**) (PEG = polyethylene glycol) in different solvents (blue = PBS, green = DMSO, orange = DMF, red = EtOH, and black = MeOH). "H" indicates H aggregates and "J" indicates J aggregates.

Cyanine dyes have a tendency to aggregate in solution through plane-to-plane stacking (parallel aggregates) or through an end-to-end arrangement (head-to-tail). The transition to the upper state in parallel aggregates leads to a hypsochromic (red) shift and these groups of molecules are called H aggregates, whereas the transition to a lower state in a head-to-tail arrangement leads to a bathochromic (blue) shift and these assemblies are called J aggregates.[9,57–62] The absorption spectra of the new aminocyanine dyes show that they have low tendency to aggregate because their main absorption bands correspond to the monomeric species. However, evidence for the formation of limited aggregation can be found in the absorption spectra of some compounds. Close analysis shows that the type and amount of aggregation depends significantly on the nature and the length of the linker, dye–$(CH_2)_5$–COOH (**8**) shows both H and J aggregates, whereas dye–$(CH_2)_{10}$–COOH (**9**) shows only H aggregates (Figure 1). Dye–$(CH_2)_6$–NHBoc (**10**) forms only J aggregates and dye–PEG–COO*t*Bu (*t*Bu = *tert*-butyl) (**11**) shows no evidence of aggregation (Figure 1, for all other spectra see the Supporting Information).

Next, we investigated the fluorescence properties of the new aminocyanine dyes. All dyes show fluorescence in the NIR region around 754 nm. Contrary to the absorption spectra, the linkers and the terminal groups do not have a significant influence on the emission wavelength (Figure 2). The dyes have high quantum yields, being higher than ICG (0.078) for all new dyes. Dye–PEG–COO*t*Bu (**13**) is the dye with the highest quantum yield (0.16), twice as high as ICG, which is probably due to the presence of some aggregates

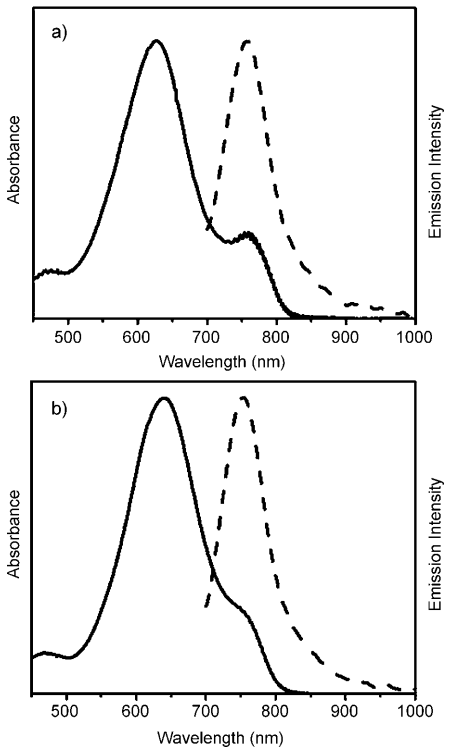


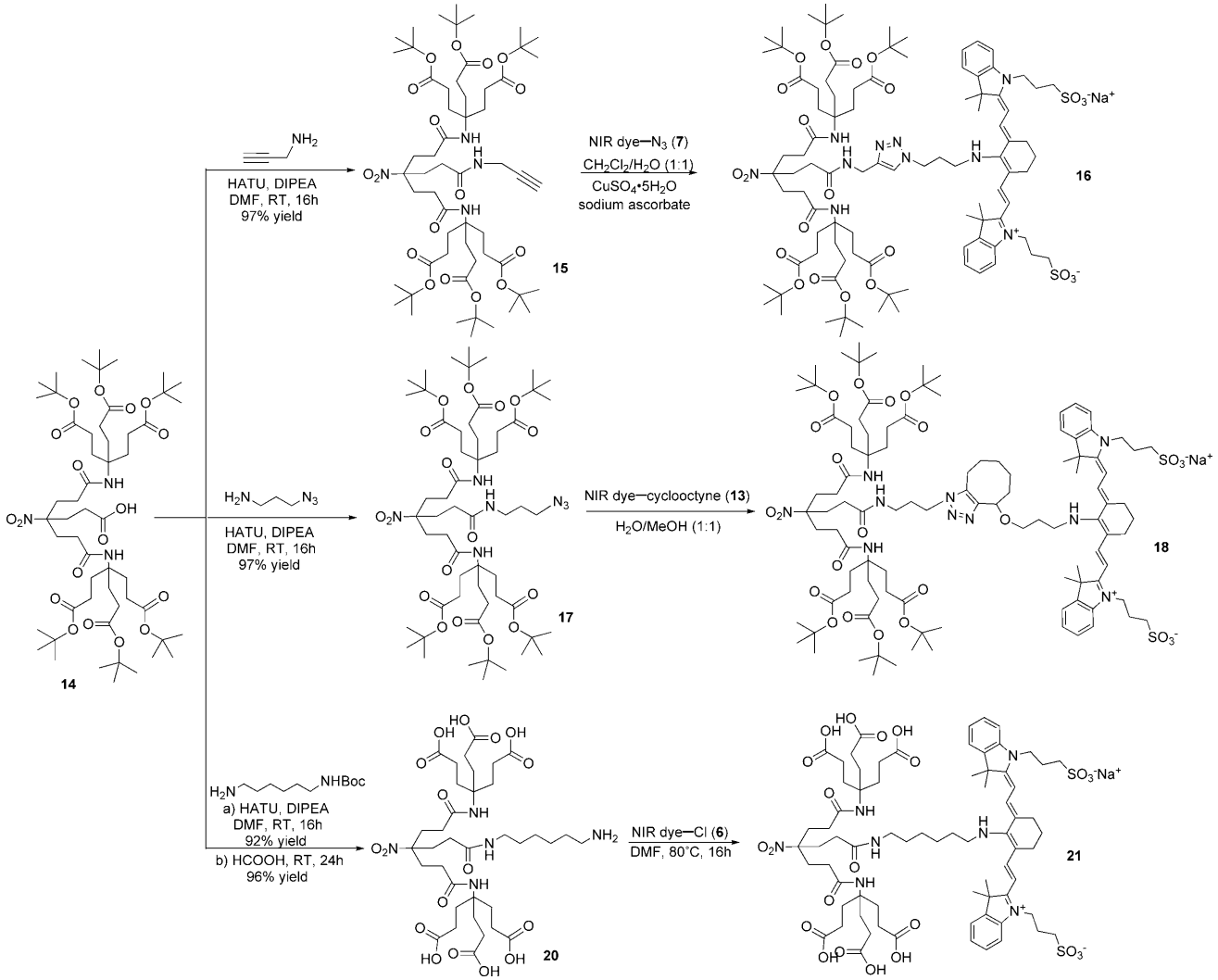
Figure 2. Absorption (solid line) and emission (dash line) spectra of a) dye–$(CH_2)_5$–COOH (**8**) and b) dye–PEG–COO*t*Bu (**11**).

for all other dyes resulting in some self-quenching (**13** does not show any kind of aggregation). The fluorescence spectra of all new dyes can be found in the Supporting Information.

Introduction of the aminocyanine dyes into one branch of AB_6C_1-type dendrons: Cyanine dyes were selectively attached to one branch of AB_6X_1 dendrons (A = NO_2; B = $COOtBu$ or COOH; X = C≡CH, = N_3, or NH_2) through three different synthetic approaches: CuAAC, SPAAC, and direct $S_{RN}1$. Multifunctional dendrons were synthesized from dendron **14**[31] containing a nitro group at the focal point, six protected acids and one free acid group as described in Scheme 6.

The coupling reaction between the acid group of **14** and propargylamine afforded dendron **15**, which contains one alkyne group, in 97% yield. The CuAAC reaction between dendron **15** and dye–azide **7** by using $CuSO_4$/sodium ascorbate in CH_2Cl_2/H_2O (1:1) afforded dendron–dye **16** as a deep blue waxy product in 82% yield. Dendron **16** was characterized by mass spectrometry showing its molecular ion peak $[M-Na]^{2-}$ at *m/z* 935.0 (*m/z* calcd for $(C_{96}H_{145}N_{10}O_{23}S_2)^{2-}$: 935.0. The deep blue coloration of **16** in the solid state slowly changed to deep brown after 24 h, and the mass spectrum no longer showed the molecular ion peak of **16**. The color change and the disappearance of the molecular ion peak in the mass spectra suggest that compound **16** degrades over time, which could have been promoted by the presence of copper traces from the CuAAC reaction.[25,33]

The reaction between the acid group of **14** and 3-aminopropyl azide by using HATU yielded dendron **17** containing one azide group in 97% yield. SPAAC between **17** and **13** was carried out by mixing both products in H_2O/MeOH (1:1) at room temperature. The reaction was followed by mass spectrometry and was complete after 48 h. After purification by dialysis, dendron–dye **18** was obtained as a deep blue waxy product and was characterized by mass spectrometry through its molecular ion peak $[M-Na]^{2-}$ at *m/z* 998.3 (*m/z* calcd for $(C_{104}H_{159}N_{10}O_{24}S_2)^{2-}$: 998.1).



Scheme 6. Attachment of NIR dyes to one branch of multifunctional dendrons by using CuAAC, SPAAC, or $S_{RN}1$. HATU = 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine.

Contrary to compound **16** synthesized by CuAAC, the dye–dendron conjugate **18** synthesized by SPAAC was stable in the solid state even after nine months of storage.

The coupling reaction between **14** and *N*-Boc-1,6-hexanediamine, followed by the deprotection of both the Boc-protected amine group and the *tert*-butyl-protected acid groups by using formic acid, afforded dendron **20** containing six acids and one amine group. The $S_{RN}1$ reaction between **20** and **6** was carried out in DMF at 80 °C for 12 h yielding dye–dendron **21** that was characterized by its molecular ion peak $[M-Na]^-$ at *m/z* 1494.4 (*m/z* calcd for $(C_{72}H_{100}N_7O_{23}S_2)^-$: 1494.6).

Attachment of NIR dyes to the focal point of dendrons: We decided to attach a NIR dye to the focal point of a dendron, leaving the peripheral functional groups available for further functionalization with targeting groups and/or drugs. Reduction of the nitro group at the focal point of the polyamide dendrons affords **22** suitable for $S_{NR}1$ reaction with **6**. Our first attempt to attach **6** to the focal point of **22** resulted, after 24 h of heating at 80 °C in DMF, in no reaction with the mass spectrum of the mixture showing only starting materials. The solution was heated for an additional 48 h but no reaction was observed and the expected compound **23** was not obtained. We rationalized that the sterically bulky *tert*-butyl groups on the dendrimer precluded the attachment of **6** to the amine group at the focal point. Thus, two different synthetic strategies to attach the NIR dye to the focal point of the dendrons were designed: 1) removal of the *t*Bu groups prior to the attachment of the dye and 2) introduction of a linker between the focal point and the NIR dye.

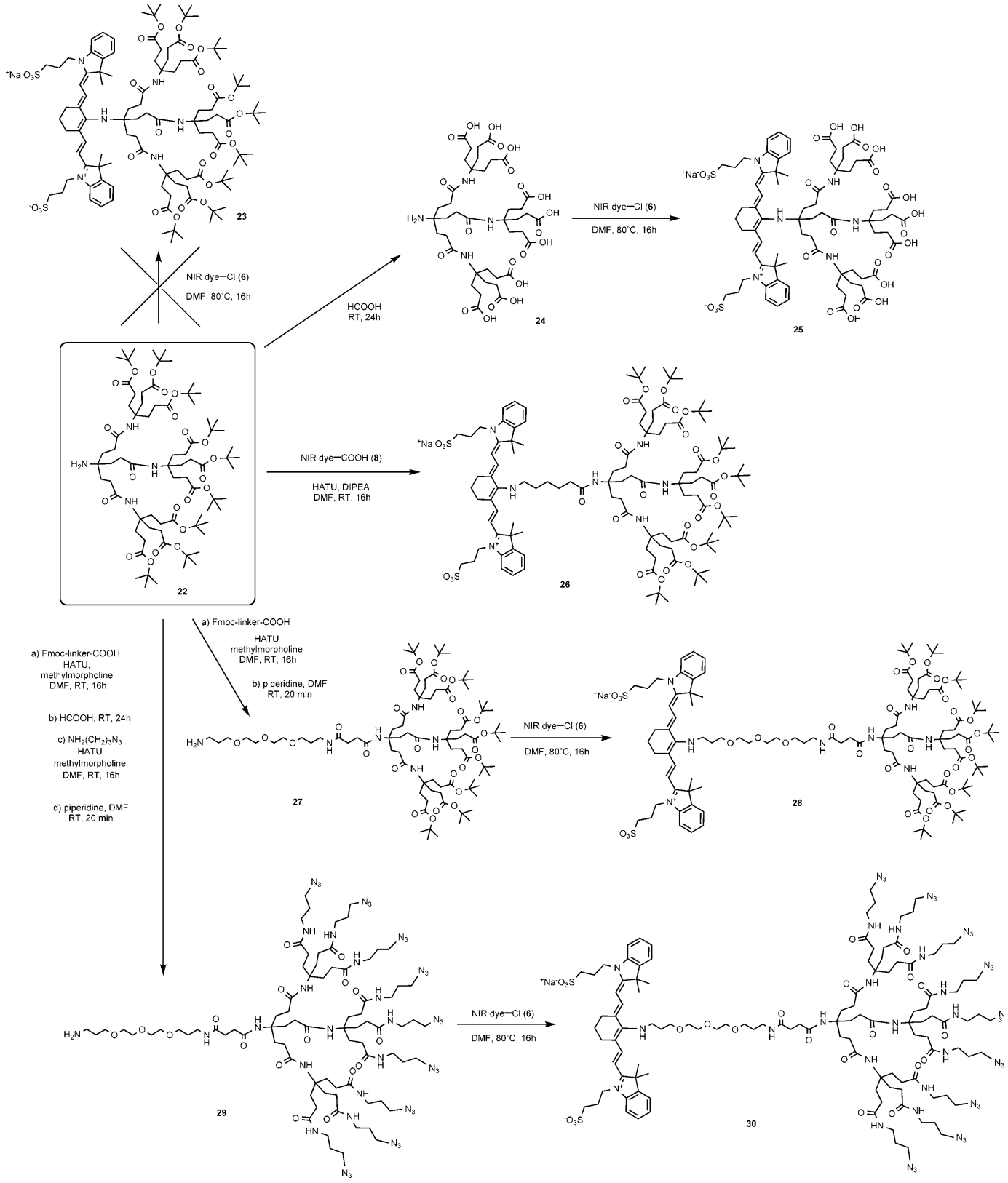
The *t*Bu groups of **22** were successfully removed by using formic acid, affording dendrimer NH_2–$(COOH)_9$ (**24**) that upon reaction with **6**, yielded dye–dendron **25** (Scheme 7). Following the second strategy, we coupled the amine group of **22** with the acid group of dye **8** that contains an alkyl linker by using HATU and DIPEA, affording dye–dendron **26** (Scheme 7). Furthermore, we introduced an ethylene glycol linker at the focal point of **22** affording dendrons NH_2–linker–$(COOtBu)_9$ (**27**) and NH_2–linker–$(N_3)_9$ (**29**), which were submitted to $S_{RN}1$ reaction with **6** resulting in the formation of the dye–dendrons **28** and **30**, respectively (for experimental details and characterization data of all dendrons and dye–dendrons, see the Supporting Information).

Optical properties of the aminocyanine dyes and the dye–dendron conjugates: The optical properties of the new dendron–dye conjugates differ significantly from each other, demonstrating that the structure and the terminal groups of the dendron dictate the dyes' behavior in solution. Dendrons dye–$(COOtBu)_6$–NO_2 (**18**) and dye–$(CH_2)_5$–$(COOtBu)_9$ (**26**) show no significant aggregation, the major absorption band corresponding to their monomeric species (Figures 3a and d). Because these two dye–dendron conjugates have a low tendency to aggregate in solution, they present optical properties similar to the aminocyanine dyes before the attachment to the dendron, including high quantum yields and large stokes shifts (Table 2). Dendron dye–$(COOH)_6$–NO_2 (**21**) shows a significant amount of monomeric species and a narrow band at higher wavelength that corresponds to J aggregates. The amount of J aggregates in this case is solvent dependent being lower in aqueous media than in alcoholic solvents (Figure 3b). The high quantum yield of this compound (0.11) is due to the presence of monomeric species and J aggregates that usually present high quantum yield and the absence of H aggregates that are known to cause self-quenching (Figures 3b and 4a, and Table 2). Dendron dye–PEG–$(COOtBu)_9$ (**28**) has a high degree of organization in solution showing only J aggregates (Figure 3e). This results in a relatively low stokes shift (21 nm) (Figure 4c), which is typical for J aggregates. The quantum yield for this compound is relatively high (0.064) being close to that of ICG (0.078). Dendron–dye conjugates **25** and **30** show stochastic mixtures of H and J aggregates with no evidence for the presence of any monomeric species in solution (Figures 3c and f). Because the H aggregates absorb in the visible region, **25** and **30** present different colors in solution. Solutions of **25** in PBS, DMSO, DMF, EtOH, and MeOH are brownish/orange. Compound **30** presents a pink coloration in PBS, EtOH, and MeOH, and an orange coloration in DMF and DMSO. The difference in the coloration of **30** in these two groups of solvents can also be observed in its absorption spectra (Figure 3f), and suggests that different types of H aggregates are formed in protic and aprotic solvents. The presence of a high amount of H aggregates causes self-quenching of the emission spectra of **25** and **30** (Figure 4b and d, and Table 2) resulting in much lower quantum yields than all the other dyes and dye–dendron conjugates (0.024 for **25** and 0.027 for **30**).

Table 2. Optical properties of the dendron–dye conjugates.

Dye	Absorption[a]					Emission[b]	
	PBS λ [nm] (ε)	DMSO λ [nm] (ε)	DMF λ [nm] (ε)	EtOH λ [nm] (ε)	MeOH λ [nm] (ε)	λ [nm]	ϕ_F[c]
18	639 (9600)	636 (20400)	618 (17900)	628 (21100)	627 (27400)	749	0.12
21	615 (27400) 772 (29200)	651 (29600) 793 (46800)	643 (18300) 790 (32300)	653 (36600) 784 (59600)	645 (41000) 780 (56800)	773	0.11
25[d]	764 (8500)	782 (14000)	778 (14600)	772 (16700)	765 (17600)	792	0.024
26[e]	–	637 (7300)	606 (800)	623 (2700)	627 (6700)	756	0.11
28	774 (30300)	797 (40000)	794 (40000)	786 (54000)	783 (56700)	804	0.064
30[d]	753 (8200)	773 (8200)	769 (4300)	763 (12400)	760 (12000)	758	0.027

[a] The absorption spectra were recorded at 25 °C and ε is expressed in $M^{-1}cm^{-1}$ at the maximum of the highest peak. [b] Excitation wavelength was 632 nm for all compounds. [c] Relative fluorescence quantum yields (ϕ_F) were determined in methanol by using the known quantum yield of ICG as a reference ($\phi_F = 0.078$ in methanol).[11] [d] Only the band in the NIR region is indicated. [e] Compound **26** is not soluble in aqueous media.



Scheme 7. Attachment of the NIR dye to the focal point of dendrons. Fmoc = fluorenylmethoxycarbonyl.

Cytotoxicity of the dye–dendron conjugate dye−PEG−$(N_3)_9$ (30): As our ultimate goal was the application of the NIR aminocyanine dyes as imaging agents in theranostics, it is essential to evaluate the cytotoxicity of the dye–dendron con-

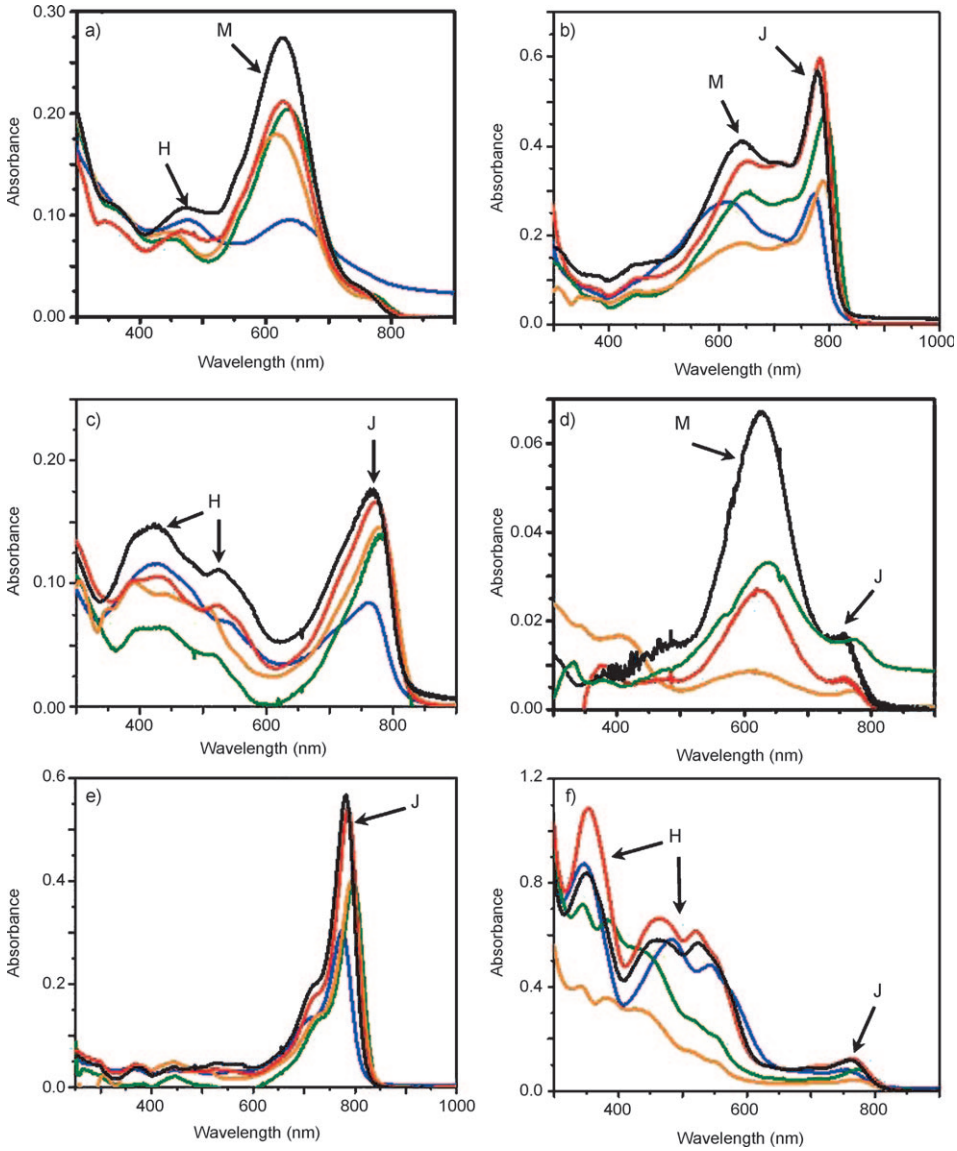


Figure 3. Absorption spectra of a) dendron dye–$(COOtBu)_6$–NO_2 (**18**) synthesized by SPAAC, b) dendron dye–$(COOH)_6$–NO_2 (**21**), c) dendron dye–$(COOH)_9$ (**25**), d) dendron dye–$(CH_2)_5$–$(COOtBu)_9$ (**26**), e) dendron dye–PEG–$(COOtBu)_9$ (**28**), and f) dendron dye–PEG–$(N_3)_9$ (**30**) in different solvents (blue = PBS, green = DMSO, orange = DMF, red = EtOH, and black = MeOH). All spectra were taken at concentrations of 1×10^{-5} M at 25 °C. "M" indicates monomeric species, "H" indicates H aggregates and "J" indicates J aggregates.

jugates. Thus, we evaluated the toxicity of **30** in human brain glioblastoma cells (T98G cell line) that were grown in the presence of different concentrations of **30**. After 72 h of incubation, living cells were quantified by using the WST-1 proliferation assay, and Figure 5 shows the results obtained for the proliferation assays for 0.5, 1.0, 5.0, 10, and 50 μM of **30**.

The proliferation assay demonstrates that even after an incubation time of 72 h, **30** does not present significant toxicity for T98G cells at concentrations up to 50 μM. These results show that besides the presence of aggregates in solution, the dye–dendron conjugate **30** is not toxic to these cells, which is encouraging for pursuing our studies on the use of these aminocyanine dyes as imaging agents in dendritic drug delivery systems.

Conclusion

In this manuscript, we report the synthesis of aminocyanine dyes containing useful functional groups such as acids, azides, and cyclooctyne groups. The aminocyanine dyes can be attached to polyfunctional dendrons by using CuAAC, SPAAC, peptide coupling, or direct $S_{NR}1$ reactions. The aminocyanine dyes and dendron–dye conjugates were obtained in high yields and showed good to excellent chemical and optical stabilities. The optical properties of the new compounds were studied by UV/Vis and fluorescence spectroscopy. All compounds show fluorescence in the NIR region. It was observed that the formation of H and J aggregates in solution strongly depends on the nature and length of the linker and the functional group on the aminocyanine dyes, as well as the dendron structure and terminal groups in the dye–dendron conjugates. Finally, cytotoxicity studies of the dye–dendron conjugates demonstrate that these materials are non toxic, which is an encouraging result for pursuing our studies on the use of these aminocyanine dyes as imaging agents in dendritic drug delivery systems.

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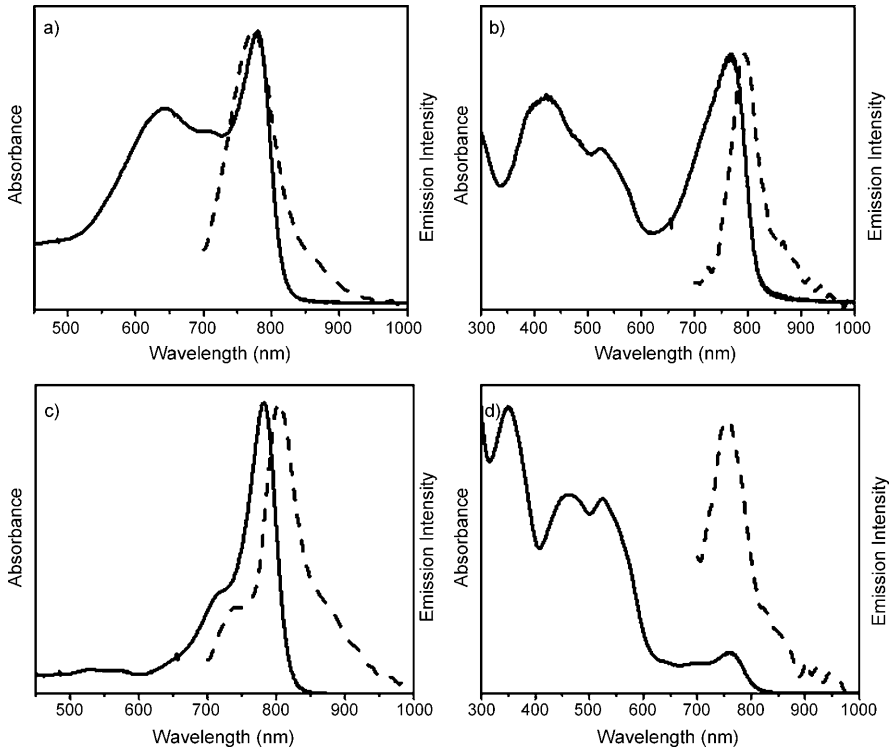

Figure 4. Absorption (solid line) and emission (dash line) spectra of a) dendron dye–$(COOH)_6$–NO_2 (**21**), b) dendron dye–$(COOH)_9$ (**25**), c) dendron dye–PEG–$(COOtBu)_9$ (**28**), and d) dendron dye–PEG–$(N_3)_9$ (**30**).

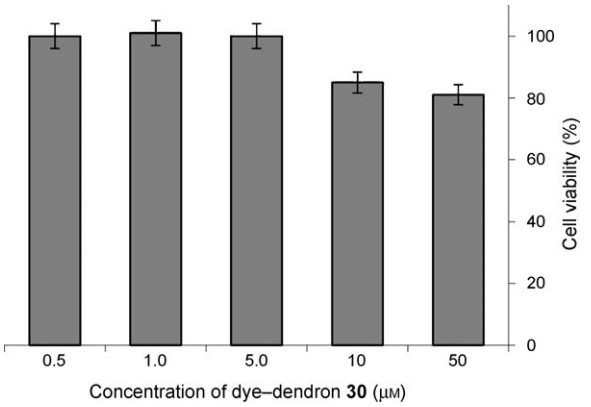

Figure 5. Results obtained for the proliferation assays carried out on human brain glioblastoma cells (T98G cell line) incubated for 72 h, in the presence of 0.5, 1.0, 5.0, 10, and 50 μM of **30**. *Y*-axis values correspond to percentage of living cells (the lowest concentration of the dye–dendron conjugate was considered as 100% of living cells). For each concentration, the proliferation assays were carried out in triplicate and the grey columns represent the average of the three assays.

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