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# A Versatile Catalyst-Free Perfluoroaryl Azide-Aldehyde-Amine Conjugation Reaction



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# A Versatile Catalyst-Free Perfluoroaryl Azide-Aldehyde-Amine Conjugation Reaction

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**A tri-component reaction, involving an electrophilically-activated perfluoroaryl azide, an enolizable aldehyde and an amine, reacts readily at room temperature without any catalysts in solvents including aqueous conditions to yield a stable amidine conjugate. The versatility of this reaction is demonstrated in the conjugation of an amino acid without prior protection of the carboxyl group, and in the synthesize antibiotic-nanoparticle conjugates.**

Efficient conjugation chemistry is indispensable in the development of molecular conjugates and functional nanomaterials in many applications. Searching for conjugation chemistries that are versatile, can accommodate a wide variety of ligands under mild conditions, do not require any special catalyst and are straightforward to carry out is a continuing challenge.[1] In this context, azide-based ligation reactions have become widely adopted, primarily due to the compatibility of the reactions with many reagents and conditions, as well as the ease of introducing the azide functionality.[2] The Cu- or Ru-catalyzed azide-alkyne click reactions (Cu/RuAAC), yielding triazoles, have extended the high reactivity and chemo-selectivity of azides, and provide a fast, clean and mild conjugation methodology.[3] An issue in the practical use of these azide-based conjugation reactions is the requirement of specific catalysts (e.g., $Cu^{I}$), which can be difficult to remove and thus result in concerns of cytotoxicity. Approaches to catalyst-free reactions have primarily focused on activating the dipolarophiles. For example, strained alkynes,[4] peptidyl phosphoranes,[5] nitrones, and tetrazines undergo fast catalyst-free cycloaddition reactions, which have found utilities in many conjugation applications.[6]

Activation of the azide is an alternative strategy to achieve metal-free transformations, but only a few reactions have been explored for conjugation reactions.[7] For example, electron-deficient phenyl azides were reported to undergo accelerated cycloadditions with electron-rich dipolarophiles.[5, 7a-i] Acyl azides react with amines under basic conditions to form amides, a reaction that was applied in peptide coupling.[7k] However, acyl azides readily decompose at >40 °C, which limits their wide use. Electron-deficient sulfonyl azides can couple with thioacids or norbornenes in high efficiency, which was employed for conjugation.[7l-n]

Perfluoroaryl azides (PFAAs) are another class of electron-deficient azides owing to the presence of multiple fluorine atoms. This electrophilic activation accelerates the reactions of PFAAs by orders of magnitude compared to their non-fluorinated counterparts, and enables new reactions of PFAAs that are unique or impossible with the non-fluorinated analogs, such as azide-thioacid amidation, azide-aldehyde amidation, azide-enamine cycloadditions, Staudinger reaction, and azide-alkyne cycloaddition.[7j, 8] PFAAs have also proven valuable for the functionalization of materials and surfaces, and in photoaffinity labeling reactions via singlet perfluoroaryl nitrenes formed by photolysis or thermolysis of the azide.[9]

In this study, we report a one-step, tri-component conjugation reaction using PFAAs, which can accommodate a wide range of amine- and enolizable aldehyde-tagged structures, without the need of any catalysts or additional agents (Scheme 1). The PFAA-aldehyde-amine reaction is proposed to proceed via an azide-enamine cycloaddition reaction to form a triazoline (supported by NMR), which spontaneously decomposed to a stable amidine-linked conjugate.[10] The release of nitrogen gas as well as the formation of the stable amidine structure suggests a strong thermodynamic driving force in these conjugation reactions, a

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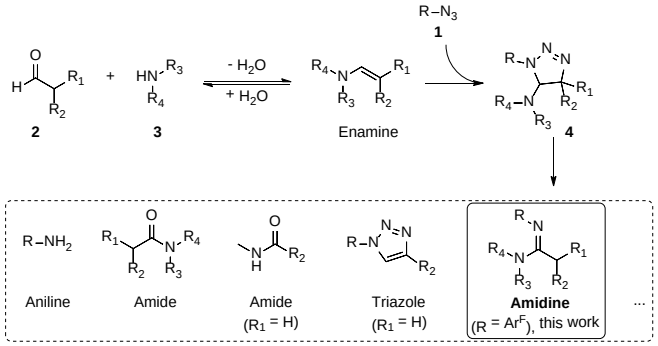


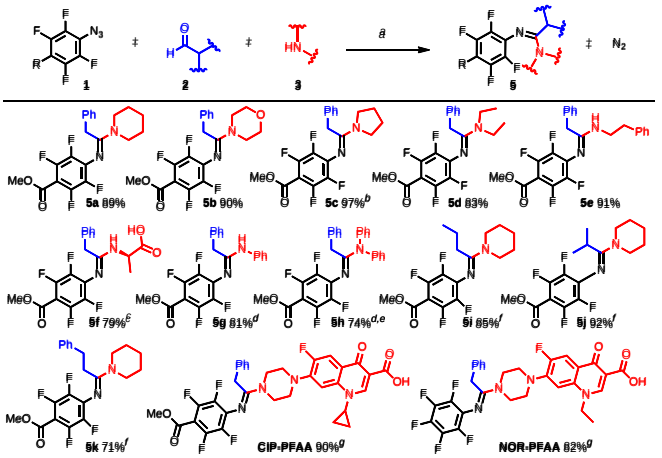

**Scheme 1.** Azide-aldehyde-amine reaction via triazoline intermediate **4.**

feature that has been emphasized in click chemistry.[11] Note that previous attempts using other azides yielded triazolines (**4**) that rearranged into a variety of products, including anilines, amides, triazoles and amidines (Scheme 1).[12] Triazoles could be selectively formed, but at harsh dehydration conditions.[13] This issue can be avoided using PFAAs, where the tri-component reaction yielded stable amidines exclusively at mild conditions. The reaction was investigated with regard to substrate scope, concentration, solvent and kinetics. The utility of this reaction was demonstrated in the conjugation of amino acid L-alinine and fluoroquinolone antibiotics, both containing un-protected carboxyl groups. The fluoroquinolone-functionalized silica nanoparticles showed enhanced antimicrobial activity compared to the corresponding molecular analog.

The model reaction was carried out by mixing methyl 4-azido-2,3,5,6-tetrafluorobenzoate (**1a**), phenylacetaldehyde (**2a**) and piperidine (**3a**) in various solvents, giving amidine **5a** in 80-92% isolated yields (Table S1, Table 1). $^1$H-NMR analysis showed fast formation of triazoline **4a** within minutes, which gradually converted to the amidine **5a** over a period of 8-24 h. Significantly, the reaction proved compatible with water, although water in principle could hydrolyze enamines and thus impair the azide-enamine cycloaddition.[14] The addition of 10-60% water to the solvent, although leading to heterogeneous emulsions, still resulted in the formation of amidine **5a** in high yields (Fig. S3). The reactions were in many cases even faster than those under homogeneous conditions. The excellent performance of this reaction under heterogeneous conditions without the need of a phase transfer agent implies that this tri-component reaction could be efficient for conjugation and nanomaterial functionalization where the reactions are often heterogeneous.

Table 1 shows the scope of amines using PFAA **1a** and phenylacetaldehyde. Secondary aliphatic amines, either cyclic or acyclic, gave amidines **5a-d** in high isolated yields. Primary aliphatic amines, for example phenethylamine, also gave clean transformations (**5e**, 91%). Based on these results, the possibility of using this reaction to modify amino acids was explored. L-Alanine, a typical *N*-terminal amino acid residue in natural proteins, was tested in its unprotected from. Results showed that L-alanine reacted smoothly in 3:1 (v:v) DMSO/water at 60 °C to give amidine **5f** in 79% yield within 2 h. The reaction was also efficient at lower concentrations (e.g., 8 mM, Table S4). Note that this reaction does not require the

**Table 1.** Reaction scope of amines and aldehydes.




[a] Reaction conditions: **1** (0.100 M), **2** (0.105 M), **3** (0.105 M), 1 mmol scale, MeOH, 25 °C, 12 h, isolated yield. [b] DMF, under $N_2$. [c] **1** (0.05 M), **2** (0.075 M), *L*-alanine (0.075 M), DMSO/$H_2O$ 3:1 v/v, 60 °C, 2 h. [d] **2** (0. 26 M), **3** (0.26 M), 40 °C, 24 h. [e] 5 d. [f] 40 °C, 2 h. [g] **1** (0.025 M), **2** (0.025 M), **3** (0.012 M), 2 mmol scale, acetone, 25 °C, 48 h.

protection of the carboxyl group, and thus provides a straightforward method for the modification of amines in the presence of free carboxyl groups. In addition, aryl amines showed good reactivity, albeit their reactions were slower than those of aliphatic amines. For example, the reaction with aniline was accomplished at 40 °C within 24 h to give amidine **5g** in 81% yield. Nucleophilically-deactivated diphenylamine also worked, giving amidine **5h** in 74% yield after 5 d at 40 °C. These results demonstrate the potential of this reaction in the conjugation and modification of aromatic amines, which are important motifs in, e.g., pigments and dyes.

Different aldehydes were screened against PFAA **1a** and piperidine (Table 1). Enolizable aldehydes generally underwent clean and fast cycloaddition in various solvents. Compared to triazoline **4a** formed from phenylacetaldehyde, the cycloaddition products formed from other aldehydes, such as butyraldehyde, displayed slower rearrangement to the corresponding amidines. The rearrangement of these triazolines was however significantly accelerated in polar solvents such as methanol. The optimal protocol for aldehydes (other than phenylacetaldehyde) was in methanol at 40 °C, where amidines **5i-k** were obtained in high yields within 2 h. The reaction was highly chemoselective, where other carbonyl compounds including non-enolizable aldehydes, ketones and hemiacetals were completely inert under these conditions.

These amidine conjugates show good stability. Due to the highly electron-withdrawing perfluoroaryl group, the acyclic amidine bond were tested to be near-neutral. These amidines showed high stability, being resistant to thermal heating or acid/base treatment (pH 1-10). To be noted, the derivative **5f** suffered from a slow hydrolytic decomposition of the amidine bond when heated under highly concentrated condition or in solid state, but was fairly stable in dilute solutions for a long time.

For a conjugation reaction to operate with high efficiency, fast kinetics is a key factor, in particular at relatively low ligand concentrations. In the PFAA-aldehyde-amine reaction, the triazoline formation is the key step for accomplishing the conjugation. In this context, the PFAA-aldehyde-amine reaction ([azide] = 0.1 M, 2-12 h) is faster than the CuAAC click reaction ([azide] = 0.25-0.5 M, 12-24 h) under similar conditions.[3a] In addition, the tri-component reaction can be accelerated by increasing the concentration of the aldehyde or the amine. When either aldehyde or amine were used at ≥20 equiv. of the azide (Fig. S5), the reaction was completed within 1 h at 10 mM PFAA concentration. This reaction profile is very close to the bimolecular azide-enamine cycloaddition (rate constant: 0.17 $M^{-1}$ $s^{-1}$ in MeOH at 22 °C, Fig. S1), which is comparable with many strain-promoted azide-alkyne cycloaddtions (0.001 ~ 1.0 $M^{-1}$ $s^{-1}$).[15] These features are likely due to the favorable enamine formation, especially when conjugated enamines are produced, even when the reactants were at concentrations as low as 0.1-10 mM.[14]

The transformation was next applied to immobilizing the antibiotics ciprofloxacin (CIP) and norfloxacin (NOR) onto nanoparticles. Nanotherapeutics, prepared by formulating drugs into nanoparticle forms, have been shown to increase the drug titer at or inside the bacterium, resulting in higher killing efficacy and lower systemic toxicity.[16] Such nanotherapeutics may also overcome antimicrobial resistance by disarming the efflux pump.[17] CIP and NOR belong to the fluoroquinolone class of broad-spectrum antibiotics. Due to their extensive use, fluoroquinolone-resistant microorganisms are prevalent, posing an increasing threat to the public health, and thus new strategies such as nanotherapeutics are needed to rescue the drug efficacy.[18] Immobilization of fluoroquinolones, especially by covalent linkage, is challenging. The presence of both carboxylate and amine functionalities makes it cumbersome to use carbodiimide-mediated conjugation directly, since either the carboxyl or the amine group needs to be protected.[19]

Figure 1a shows the straightforward one-step immobilization protocol. PFAA-functionalized Stöber silica nanoparticles (**PFAA-SNPs**) of ~50 nm or ~100 nm size were synthesized.[20] Afterwards, an aqueous suspension of CIP or NOR (1.5 mM) and phenylacetaldehyde (20 equiv.) were stirred with the **PFAA-SNPs** (5 mg/mL) in acetone at room temperature. The reaction was followed by FTIR by monitoring the characteristic azide peak at 2125 $cm^{-1}$. In the case of NOR, the azide peak decreased quickly within the first 4 h, which was then followed by a slow period of up to 24 h. For CIP, the reaction proceeded through an initial stage of 48 h, followed by a slow period of up to 7 d. After conjugation, the resulting particles dispersed well in water (8-10 mg/mL), whereas the starting material **PFAA-SNPs** did not.

Following conjugation of the fluoroquinolones, the azide signal at 2125 $cm^{-1}$ decreased in the FTIR spectrum (Fig. 1b). At the same time, the peak at 1689 $cm^{-1}$, corresponding to the carboxyl group in the fluoroquinolone, was observed together with the characteristic amidine peak at 1620 $cm^{-1}$. The covalent functionalization was further supported by XPS analysis. The high resolution N1s spectrum of the **PFAA-SNPs** (Fig. 1c, top) shows peaks at 405.3 eV (-N=N=N), 401.9 eV (-N=N=N) and 399.7 eV (amide N).[21] After conjugation of ciprofloxacin, the peak at 405.3 eV disappeared, with the spectrum showing a small peak at 401.1 eV, corresponding to the amidine N, and a large peak at 399.7 eV corresponding to the $sp^3$ N atoms in **CIP-SNPs** (Fig. 1c, bottom)**.** The **CIP-SNPs** displayed moderate fluorescence (Ex/Em: 338/462 nm) (Fig. 1d). Thermogravimetric analysis (TGA) was used to estimate the amount of drug molecules attached on the nanoparticle. For SNPs of ~50 nm size, the weight loss difference before and after drug

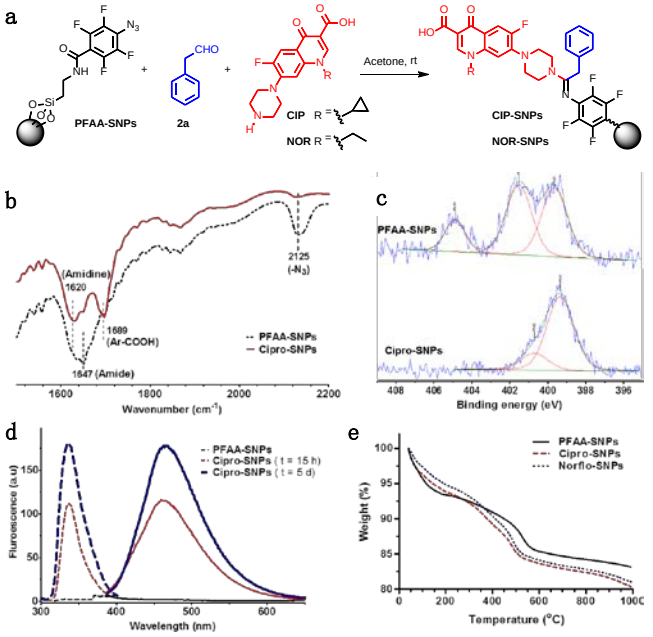



**Figure 1**. Conjugation and characterization of CIP and NOR to silica nanoparticles using PFAA-amine-aldehyde reaction. (a) The reaction scheme. (b) FT-IR spectra of **PFAA-SNPs** and **CIP-SNPs**. (c) High-resolution N1s XPS spectra of **PFAA-SNPs** and **CIP-SNPs**. (d) Emission (solid line) and excitation (dashed line) fluorescence spectra of **PFAA-SNPs** and **CIP-SNPs** at reaction time t = 15 h and 5 d. Conditions: 1 mg/mL in water. (e) TGA analysis of **PFAA-SNPs**, **CIP-SNPs** and **NOR-SNPs**. Particle size: 50 ± 15 nm by TEM.

conjugation was 2.7 ± 0.5 % for **CIP-SNPs**, and 2.1 ± 0.4 % for **NOR-SNPs** (Fig. 1e), corresponding to an immobilized ligand density of (13.4 ± 2.5) × $10^{-16}$ nmol/$nm^2$ for **CIP-SNPs**, and (10.4 ± 2.0) × $10^{-16}$ nmol/$nm^2$ for **NOR-SNPs**. These data compare well with those obtained using PFAA photocoupling chemistry (19.8 × $10^{-16}$ nmol/$nm^2$ for trehalose-modified SNPs),[20] or the CuAAC reaction (13.0 × $10^{-16}$ nmol/$nm^2$ for mannose-modified SNPs),[22] demonstrating high efficiency of the reaction.

The *in vitro* antibacterial activities of the drug-modified SNPs were evaluated against fluoroquinolone-resistant *E. coli* ORN208.[23] **CIP-SNPs** of ~50 nm size had an MIC (minimum inhibitory concentration) of 54 μg/mL, which represents circa three-fold higher killing activity than that of the molecular analog **CIP-PFAA** (Table S7). Similarly, the MIC of ~50 nm **NOR-SNPs**, 84 μg/mL, was about six-fold improvement than that of the **NOR-PFAA**. In addition, the antibacterial activity increased with decreasing particle size: ~50 nm particles had lower MIC compared to those of ~100 nm for both **CIP-SNPs** and **NOR-SNPs**.

The *E. coli* cells showed weak auto-fluorescence at 405 nm excitation (Fig. 2a). To overcome the interference from the bacteria and the fluorescence from the surface-bound CIP (Fig. 1d), fluorescein-doped SNPs (FSNPs) were used,[20, 24] and **CIP-FSNPs** (*d* ~110 nm) were synthesized following the same conjugation protocol as **CIP-SNPs**. **CIP-FSNPs** were incubated with *E. coli* ORN208 for 4 h, and the resulting samples were examined under confocal fluorescence microscopy. **CIP-FSNPs** emitted strong blue-green fluorescence at both 405 nm and 488 nm excitation, where the latter wavelength resulted in a narrower emission band centered around ~520 nm (Fig. 2c) with

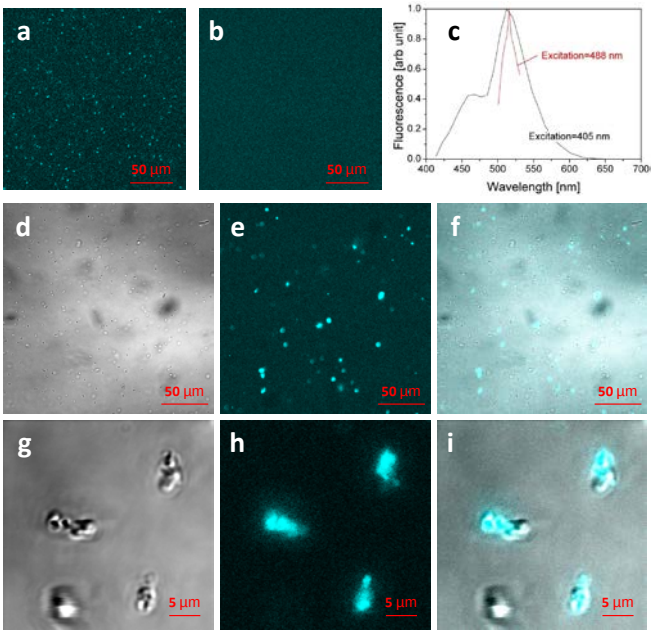



**Figure 2.** Confocal fluorescence images of *E. coli* ORN208 (a) at 405 nm excitation (Ex) showing bacteria auto-fluorescence, and (b) at Ex = 488 nm showing the absence of bacteria auto-fluorescence. (c) Fluorescence spectra of **CIP-FSNPs** at Ex = 405 nm or 488 nm. (d-i) *E. coli* ORN208 (~$10^8$ CFU/mL) incubated with **CIP-FSNPs** (0.1 mg/mL) for 4 h: bright-field (d, g), fluorescence showing pseudo color (e, h) and merged (f, i) images. Ex = 488 nm (e), or 405 nm (h).

lower interference from the autofluorescence of the bacteria (Fig. 2b). Although no targeting effect of the nanoparticles was anticipated, the merged bright-field and fluorescence images showed many particles associated with bacteria cells (Fig. 2e-f, S13). The enlarged view (Fig. 2g-i) furthermore revealed that the particles co-localized with some, or parts, of the bacterium. A video recording of the sample showed that **CIP-FSNPs** moved together with the live bacteria cells, demonstrating high stability of the particle–cell interactions (cf. Supplementary video). In contrast, **PFAA-FSNPs** without surface-bound antibiotics mainly self-aggregated as isolated giant particles.

These observations indicate that the surface-bound fluoroquinolone structures could be involved in the association of the nanoparticles with the bacterial cells, for example via charge- and/or dipole interactions with membrane proteins or phosphatidylethanolamine components of *E. coli*.[25] Such attractive interactions have been hypothesized to facilitate the approach and binding of fluoroquinolones to cell envelopes, which contributes to their fast cross-membrane penetration.[25] These features may suggest the participation of this class of broad-spectrum antibiotics in facilitating the penetration of nanoparticles into bacteria, given that their effective enzyme targets are intracellular.[26]

In summary, we have developed a new conjugation reaction using electron-deficient PFAAs. The reaction can be straightforwardly carried out by mixing an aldehyde, an amine and a PFAA in a solvent at room temperature, with the amidine product formed cleanly without the addition of any catalysts. The reaction is compatible with various solvents, also tolerating the addition of a large percentage of water. It shows high selectivity, is efficient even at low concentrations (down to 0.1 mM), and proceeds with a wide range of amines and enolizable aldehydes. The reaction can also be used to selectively modify amines in the presence of free carboxylic acid groups, an advantage when, for example, derivatizing peptides and amino acids. Furthermore, we have successfully applied the efficient reaction to conjugate the antibiotics ciprofloxacin and norfloxacin to SNPs. The resulting fluoroquinolone-nanoparticle conjugates showed improved antibacterial activities, compared to the unconjugated molecular analogs, in a particle size-dependent fashion. Using fluorescence imaging, we observed enhanced interactions of those fluoroquinolone-nanoparticles with *E. coli* cells, a feature that may contribute to their enhanced antibacterial activities. These results demonstrate the potential of this catalyst-free and highly versatile PFAA-aldehyde-amine reaction for use in a wide range of applications including organic synthesis, bioconjugation and materials functionalization.

## Conflicts of interest

There are no conflicts to declare

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