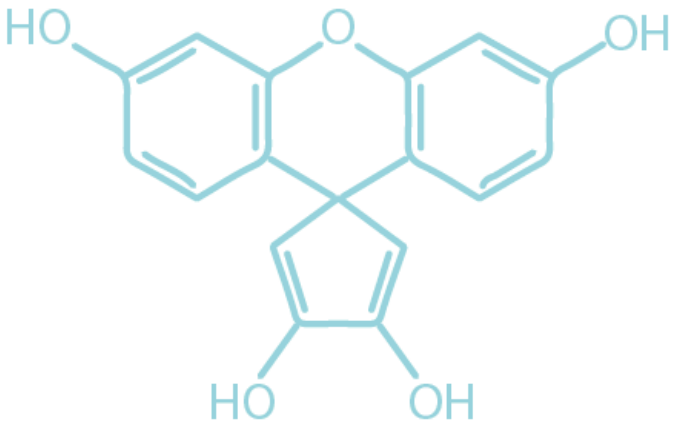
Computational Exploration of Molecules to Enhance Fluorescence Lifetimes for Superior Forensic Utility

Emilia Zhang



ABSTRACT

Latent blood visualizers are used in forensic science to detect traces of blood at crime scenes by reacting with it chemically to release an indicator, improving visualization of biofluids for documentation and analysis. However, many current latent blood visualizers—like luminol, leucomalachite green, leucocrystal violet, and fluorescein—require alternative light sources and environments, or have limited fluorescence lifetimes. This study focuses on the latter, exploring changes in molecular structure to increase fluorescence lifetime by examining molecules' excitation energies, finding tetrasubstituted carbon bonds to be most effective in raising fluorescence lifetime.

Key words: Blood, Fluorescence, Python, Forensics

INTRODUCTION

Forensic scientists use latent blood visualizers to detect traces of blood that have been cleaned and since dissipated at a crime scene. These visualizers react with the blood and release some form of an indicator, making it easier for the evidence to be analyzed and documented. The first and most common visualizing technique is the use of luminol. A solution composed of luminol, hydrogen peroxide, and a basic solution is sprayed onto surfaces suspected to contain traces of blood. The basic solution causes the luminol to form an anion, allowing it to react with hydrogen peroxide, the oxidizing agent. The iron in the hemoglobin in blood acts as a catalyst for this reaction, resulting in chemiluminescence. However, luminol also has its disadvantages: many cleaning solutions will also react with the solution, causing both false positives and negatives, and its fluorescent lifetime is short, allowing visualization only in complete darkness. Longer fluorescent lifetimes are important in accurately documenting and analyzing latent blood stains. Other visualizers, including Bluestar and leucocrystal violet, can be used in the place of luminol to compensate for its short fluorescent lifetime, but they, too, have similar limitations. This study examines fluorescent lifetimes of different compounds through their excitation energy to improve on current latent blood visualizers. While fluorescent lifetime is the focus, toxicity, synthetic feasibility, and solubility of molecules should also be considered.

Luminol is one of the most preferred and well-known latent blood visualizers in literature. As a heme-reacting solution, it reacts only with blood, and not other bodily fluids, although small traces of blood in fluids like saliva or urine may be detected. Because it reacts via oxidation, luminol may also catalyze other substances, most notably cleaning agents like bleach that are often present at crime scenes. This causes the possibility of both false positives and negatives, though trained crime scene investigators are said to be able to differentiate luminol reactions with blood versus bleach.[1] Regarding DNA analysis, whether or not luminol degrades DNA has been greatly contested: there is literature showing that luminol does not interfere with subsequent DNA testing[2], but in-field results can differ. Another limiting factor in the use of luminol is its fluorescence. When reacting with blood, its chemiluminescence only lasts around 30 seconds and is only visible in dark environments. This makes it difficult to accurately document and analyze luminol stains.

Other solutions are often used in substitution due to luminol's short fluorescence lifetime, namely Bluestar, a proprietary luminol-based solution. Bluestar is said to be able to luminesce for several minutes, and have a brighter luminescence so it does not require completely dark environments. Additionally, it does not degrade DNA evidence, though in-field results may vary from what is claimed.[3] Because it is chemically similar to luminol, it reacts through oxidation and has similar false positives, however, it is also claimed that Bluestar widens the observable difference between luminol-blood and luminol-bleach reactions.

Leucomalachite green and leucocrystal violet are also substitutions to luminol. Instead of producing a chemiluminescent glow like luminol-based solutions, these latent

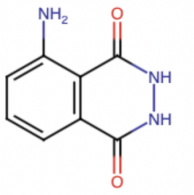
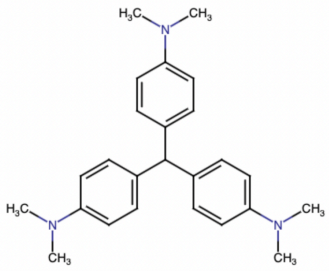
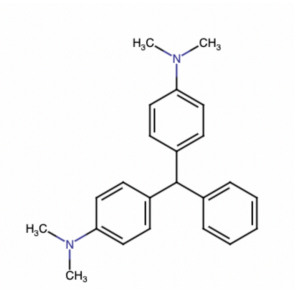
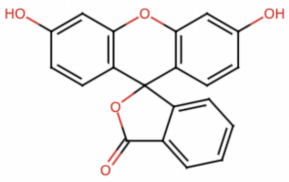
blood visualizers turn green and violet, respectively, when reacting with blood. However, because they, too, react by oxidizing when catalyzed by hemoglobin, they have similar false positives and negatives as luminol-based visualizers. Additionally, leucocrystal violet has been shown to eventually react with all of its surroundings due to light and oxygen.[4] Though the subsequent use of the chemical amido black has been suggested to counteract this effect, it nevertheless hinders the ability to efficiently photograph and document reacted stains for analysis.[4]

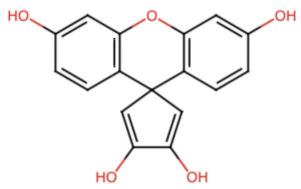
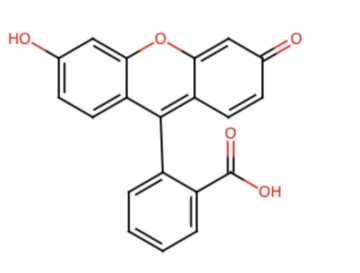
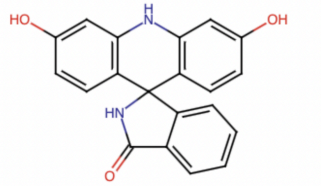
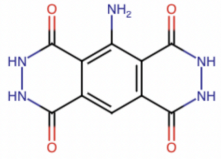
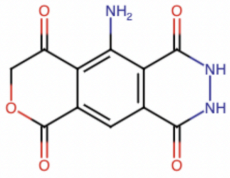
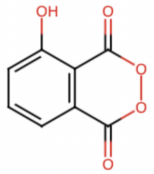
Fluorescein is another alternative latent blood visualizer, considered more DNA safe than luminol.[5] Its fluorescence can last several minutes and is visible to the naked eye, but requires an alternate light source to observe and document for accurate analysis.[6] Furthermore, fluorescein is a double application reagent, requiring the application of hydrogen peroxide after itself; this causes reagent running which may distort bloodstain patterns, further making it difficult to later analyze.[6] While many latent blood visualizers may improve on luminol's fluorescence ability and lifetime, they also have shortcomings that affect important in-field applications, documentation, and analysis. Described here is a computational exploration into structural analogs of known latent blood analyzers, with the goal of addressing these pitfalls.

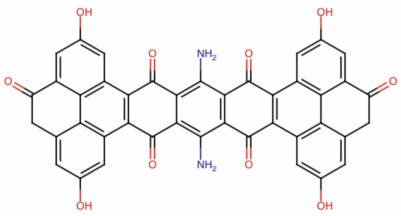
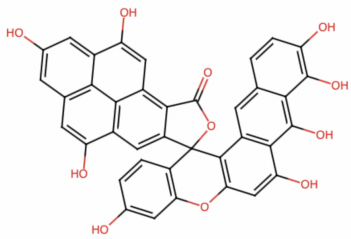
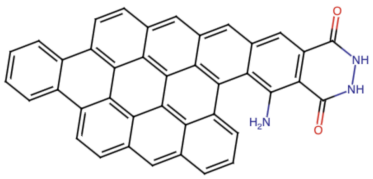
METHODS

Fluorescence excitation energy calculations: Excitation energies for proposed molecules were tested computationally using RDKit[7] and PySCF.[8] Python script is included in the appendix. In short, molecules were drawn using Protein Data Bank chemical sketch tool[9], and exported via SMILES nomenclature. The SMILES string associated with the drawn molecule was inserted into a function titled, "smiles_to_cartesian" which converted the molecule into a three dimensional object with all chemical bonds tied to xyz cartesian coordinates. These coordinates were then initialized as a Kohn-Sham density functional theory (DFT) calculation[10], providing electron structure and density information. Finally, a time-dependent DFT calculation was performed based on the Kohn-Sham calculation, providing the excitation energy for a given molecule. DFT calculations were performed under an assumed vacuum and are agnostic to solvent effects of fluorescence.

RESULTS

Local Identifier	SMILES Identifier	Structure	Excitation Energy (eV)
A	Luminol: C1=CC2=C(C(=C1)N)C(=O)NNC2=O		[1.1, 2.8, 3.5]
B	Leucocrystal Violet: CN(C)C1=CC=C(C=C1)C(C1=CC=C(C=C1)N(C)C)C1=CC=C(C=C1)N(C)C		[0.7, 2.0, 5.4]
C	Leucomalachite Green: CN(C)C1=CC=C(C=C1)C(C2=CC=CC=C2)C3=CC=C(C=C3)N(C)C		[0.3, 1.1, 3.4]
D	Fluorescein: C1=CC=C2C(=C1)C(=O)OC23C4=C(C=C(C=C4)O)OC5=C3C=CC(=C5)O		[3.4, 3.9, 5.3]

E	OC1=CC2(C=C1O)C1=C(OC3=C2C=CC(O)=C3)C=C(O)C=C1		[4.8, 4.9, 5.3]
F	Fluorescein isomer: OC(=O)C1=CC=CC=C1C1=C2C=CC(=O)C=C2OC2=CC(O)=CC=C12		[0.2, 0.4, 0.8]
G	OC1=CC2=C(C=C1)C1(NC(=O)C3=CC=CC=C13)C1=C(N2)C=C(O)C=C1		[0.2, 0.5, 0.9]
H	NC1=C2C(=O)NNC(=O)C2=CC2=C1C(=O)NNC2=O		[1.5, 1.7, 2.3]
I	NC1=C2C(=O)COC(=O)C2=CC2=C1C(=O)NNC2=O		[0.4, 0.8, 1.3]
J	OC1=CC=CC2=C1C(=O)OOC2=O		[0.4, 0.9, 1.4]

K	NC1=C2C(=O)C3=C(C(=O)C2=C(N)C2=C1C(=O)C1=C(C2=O)C2=C4C(=CC(O)=C2)C(=O)CC2=CC(O)=CC1=C42)C1=C2C(=CC(O)=C1)C(=O)CC1=CC(O)=CC3=C21		[0.4 ,0.5, 0.6]
L	OC1=CC2=C(C=C1)C1(OC(=O)C3=C4C=C(O)C5=CC(O)=CC6=C5C4=C(C=C13)C(O)=C6)C1=C(O2)C=C(O)C2=C1C=C1C=CC(O)=C(O)C1=C2O		[2.2, 2.5, 2.7,]
M	NC1=C2C(C=C3C=C4C=CC5=C6C4=C4C3=C2C2=CC=CC3=CC7=CC=C(C8=CC=CC=C58)C6=C7C4=C23)=CC2=C1C(=O)NNC2=O		[0.8, 1.0, 1.5]

DISCUSSION

Literature has shown that size, rigidity, and the presence of aromatic regions have a positive correlation with a molecule's fluorescence lifetime. With these factors in consideration, the structures of current latent blood visualizers were modified to increase their excitation energy.

Of note—between molecules H and I, the addition of nitrogens significantly increased the molecules excitation energy, while in molecules D and G, the addition of nitrogens decreased excitation energy. Compared to D and G, molecules H and I are smaller and less rigid, relying more on its structural components for excitation energy. The hydrogens on the added nitrogens in molecule H therefore contribute more to the molecule's excitation energy. Comparing molecules D and G, for molecule G, size and rigidity contribute more to its excitation energy, so the addition of nitrogens in molecule G negatively affects its excitation energy. However, because the excitation energies calculated above were performed in an assumed vacuum, the use of different solvents, especially in the case for H and I, could change the amount of fluorescence produced.

Interestingly, in molecules M and J, larger size and presence of aromatic rings did not necessarily increase excitation energy, although increased aromaticity has been shown to increase fluorescence lifetimes. The molecules are similarly based on luminol,

though additional aromatic rings are appended to molecule M, yet the two molecules had similar excitation energy. Additionally, in comparison to luminol, molecule M's excitation energy was lower. This may be due to the size of molecule M, whose aromatic rings would distribute energy more, requiring more energy to excite than luminol.

In fluorescein, molecule D, the tetrasubstituted carbon increased the excitation energy, comparable to molecule F, which had lower excitation energy and lacked the tetrasubstituted carbon. This relationship is also observable between molecules K and L. The presence of the tetrasubstituted carbon could be increasing excitation energy because of the flexibility it provides. However, the carbon-carbon bond in molecule F may be too flexible, causing the molecule to lose excitable energy.

Molecule E produced the most excitation energy of the molecules tested. Because it is modeled after fluorescein, and shares many structural characteristics, its synthetic difficulty should be relatively similar. Moreover, molecule E contains more hydroxyl groups, increasing its solubility. While it is difficult to predict toxicity of molecules computationally[11], one could assume the molecule has similar toxicity to fluorescein, which is minimally toxic in cell culture, but harmful if ingested in humans, due to similarities in molecular structure.[12,13]

The use of fluorescein in forensic applications is limited in part from the necessity of ultraviolet light sources. However, the longer fluorescence time may outweigh the downside of a secondary light source. In terms of fluorescein's double-application requirement, molecule E may reduce or eliminate the need for a subsequent application of hydrogen peroxide as it has a higher excitation energy than that of fluorescein, and peroxide is primarily used only to enhance the fluorescence. Therefore, molecule E provides promise to be a more effective latent blood visualizer.

CONCLUSION

Comparison through literature of existing latent blood visualizers found fluorescein to be the most promising alternative to luminol, as it is considered more DNA safe and displays a longer observed fluorescence. Computational explorations performed in the study found the presence of a tetrasubstituted carbon on structural analogs to luminol proved to be especially effective in increasing the excitation energy of a molecule, resulting in an iteration of fluorescein with a tetrasubstituted carbon (molecule E) with increased fluorescence lifetimes relative to the benchmark molecules luminol and fluorescein. Computationally, it is difficult to predict a molecule's synthetic feasibility and toxicity. Wet lab experiments would need to be performed to determine if it is qualified for in-field use, a possible future direction of the study. However, fluorescein has been synthesized and is known to be minimally toxic in cell culture, suggesting the pathway for molecule E translation will be similar. If this is so, molecule E could potentially facilitate documentation and analysis of latent blood evidence, minimize error, and increase the probability of identification.

APPENDIX

```
from rdkit import Chem
from rdkit.Chem import AllChem
from pyscf import gto, scf, dft, tddft

def smiles_to_cartesian(smiles):
    mol = Chem.MolFromSmiles(smiles)
    mol = Chem.AddHs(mol)

        params = AllChem.ETKDGv3()
    AllChem.EmbedMolecule(mol, params)

    conf = mol.GetConformer()
    coordinates = ""
    for i in range(mol.GetNumAtoms()):
        atom = mol.GetAtomWithIdx(i)
        pos = conf.GetAtomPosition(i)
        coordinates += f"{atom.GetSymbol()} {pos.x:.4f} {pos.y:.4f} {pos.z:.4f}; "
    return coordinates.rstrip('; ')

######Input SMILES Below########
coords = smiles_to_cartesian("")

mol = gto.Mole()
mol.build(atom=coords, basis='sto-3g')  d

mf = dft.RKS(mol)
mf.xc = 'b3lyp'
mf.kernel()

mytd = tddft.TDDFT(mf)
excitation_energies = mytd.kernel()[0]  # Extract the excitation energies
```

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