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IMMOBILIZATION OF ORGANOPHOSPHORUS ACID ANHYDROLASE MUTANT Y212F ON SILICA NANOSPHERES

Mark A. Guelta
Steven P. Harvey
Melissa M. Dixon

RESEARCH AND TECHNOLOGY DIRECTORATE

Richard Baldwin

NANOCOMPOSIX
San Diego, CA 92111-1806

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PREFACE

The work described in this report was authorized under project no. R.0014307.133.1. This work was started in August 2014 and completed in December 2014.

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IMMOBILIZATION OF ORGANOPHOSPHORUS ACID ANHYDROLASE MUTANT Y212F ON SILICA NANOSPHERES

1. INTRODUCTION

Catalytic enzymes have the potential to protect humans against organophosphate poisoning, if they are administered before exposure to the contaminant, and they could be used to detoxify these compounds after exposure, if a therapeutic compound can be developed. In addition to effectiveness, a successful therapeutic compound would need to have a sufficient shelf life in liquid or dry form and be thermally stable. Stability and shelf life might be increased by attaching the compound to other particles, including silicon dioxide.

The purpose of this study was to determine if an enzyme that is shown to have good activity on an organophosphorus nerve agent can retain this activity and to determine at what level this activity can be retained after attachment of the enzyme to a silicon dioxide nanoparticle.

The enzyme Y212F is an engineered mutant of the organophosphorus acid anhydrolase (OPAA) with good catalytic activity on diisopropyl fluorophosphate (DFP) and pinacolyl methyl phosphonofluoridate (GD, soman). This enzyme was used as a baseline to measure changes in immobilized enzyme activity. DFP was used during binding optimization studies at nanoComposix (San Diego, CA). GD was used as the substrate at the U.S. Army Edgewood Chemical Biological Center (ECBC) to measure enzyme activity before and after stabilization on the silica nanoparticle.

2. METHODS

2.1 Production of Y212F Enzyme

The OPAA Y212F *Escherichia coli* clone was prepared by DNA2.0, Inc. (Menlo Park, CA) personnel with the amino acid sequence shown in Figure 1.

MNKLAVLYAE	HIATLQKRTR	EIIERENLDG	VVFHSGQAKR	QFLDDMYYPF	50
KVNPQFKAWL	PVIDNPHCWI	VANGTDKPKL	IFYRPVDFWH	KVPDEPNEYW	100
ADYFDIELLV	KPDQVEKLLP	YDKARFAYIG	EYLEVAQALG	FELMNPEPVM	150
NFYHYHRAYK	TQYELACMRE	ANKIAVQGHK	AARDAFFQ GK	SEFEIQQAYL	200
LATQHSENDT	PFGNIVALNE	NCAILHYTHF	DRVAPATHRS	FLIDAGANFN	250
GYAADITRTY	DFTGEGEFAE	LVATMKQH QI	ALCNQLAPGK	LYGELHLDCH	300
QRVAQTL SDF	NIVNLSADEI	VAKGITSTFF	PHGLGHHIGL	QVHDTVGGFMA	350
DEQGAHQEPP	EGHPFLRCTR	KIEANQVFTI	EPGLYFIDSL	LGDLAATDNN	400
QHINWDKVAE	LKPFGGIRIE	DNIIVHEDSL	ENMTRELELD		

Figure 1. OPAA Y212F amino acid designations.

E. coli cell cultures were grown in Luria broth (Sigma-Aldrich Company; St. Louis, MO) with 100 µg/mL of ampicillin and 0.1 mM MnCl₂ at 25 °C to the late log or early stationary phase. Cells were separated using a centrifuge at 14,000 × *g* for 15 min. Cells were resuspended in 10 mM bis-trispropane (BTP) and 0.1 mM MnCl₂ at pH 7.2 at 3 mL/g of pellet weight. Cells were lysed with a sonic nozzle using three 15 s bursts in an ice bath. Cellular debris was separated at 18,000 × *g* for 120 min. The active fraction was separated at 4 °C using an ÄTKA Express purification system (GE Healthcare Life Sciences; Uppsala, Sweden) with a Q-sepharose column (HiPrep Q FF 16/10; GE Healthcare Life Sciences) using a salt gradient of 0–0.4 M NaCl and a pH gradient of 7.2 to 6.8. The active peak was dialyzed in 10 mM BTP and 0.1 mM MnCl₂ at pH 7.2.

2.2 Conjugation of Y212F Enzyme to 80 nm Amine Terminated SiO₂

The 80 nm amine terminated SiO₂ particles (nanoComposix lot no. DAC1260) were previously fabricated at nanoComposix as a standard product (Figure 2). Before the material was used for conjugation, a quality check for size was performed using dynamic light-scattering and transmission electron microscopy (TEM), and a zeta potential was performed to check amine functionality. The conjugation of the OPAA mutant Y212F enzyme to the 80 nm amine SiO₂ (ID no. CLF0293) was performed following standard Solulink (Solulink, Inc.; San Diego, CA) conjugation chemistry. To perform this conjugation, a covalent bond was formed between an aromatic hydrazine, S-HyNic (succinimidyl 6-hydrazinonicotinate acetone hydrazone), and an aromatic aldehyde, S-4FB (succinimidyl 4-formylbenzoate), which were conjugated initially to amino groups on the enzyme and the particle of choice. To determine the success of the conjugation, the fluoride electrode assay was performed with the sample on DFP. Enzyme that was covalently bound to the SiO₂ particle as described had no DFP activity (data not shown).

A second conjugation of the Y212F enzyme to an 80 nm amine terminated SiO₂ with a longer linker was prepared following the same Solulink conjugation chemistry mentioned above for the initial conjugate (ID no. CLF0334). The longer linker, C6-S-4FB (C6-succinimidyl 4-formylbenzoate), has a chain of five carbons between the two reactive sites for conjugating free amino groups and S-HyNic. The longer linker was conjugated to the OPAA mutant Y212F enzyme with the shorter linker as previously described. A colorimetric bicinchoninic acid (BCA) assay was used to measure the enzyme bound to the particle for subsequent enzymatic activity determinations.

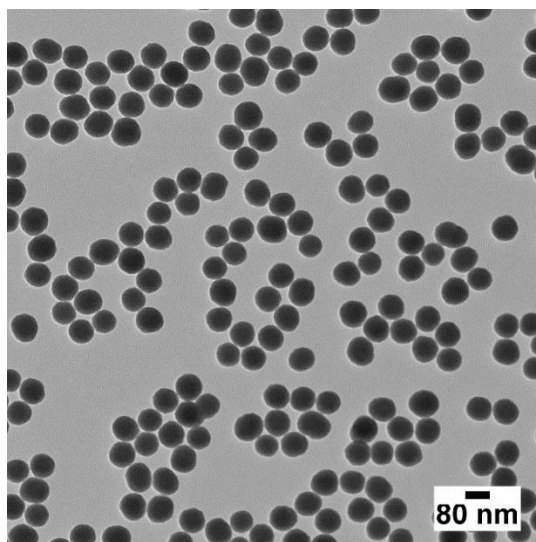


Figure 2. TEM image of 80 nm silica particles from DAC1260, which was employed for the conjugation of the Y212F enzyme.

2.3 Testing of Conjugated Enzyme Activity

The G-agent simulant DFP and agent GD, used in the study and represented in Figure 3, each contain fluoride that is released during hydrolysis. Enzymatic activity of these substrates can be measured by monitoring the fluoride concentration in solution after subtracting the spontaneous hydrolysis rate.

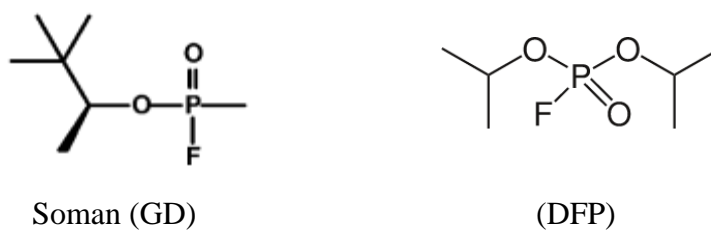


Figure 3. Chemical structures of GD and DFP.

G-agent substrates used in the study were Chemical Agent Standard Analytical Reference Material obtained from ECBC stocks.

Fluoride concentration was measured using a VWR International (Radnor, PA) Symphony probe and collected on a WTW (Wissenschaftlich-Technische Werkstätten GmbH; Weilheim, Germany) 7350 pH/Ion data logger. Assays were performed in a glass 10 mL double-wall reaction cell with circulating water at 25 °C. Activity was assayed in 50 mM BTP at pH 8.0

on a 3.0 mM substrate. Enzyme catalytic activity is expressed as the rate of fluoride ion production in micromoles per minute per milligrams of enzyme.

Sample concentrations for free enzyme in BTP solutions assayed at ECBC were measured as protein A280 using the Nanodrop 1000 software, version 3.8.1 (Thermo Fisher Scientific, Inc.; Waltham, MA). Nanospheres in suspension interfere with the Nanodrop A280 method; therefore, a BCA protein assay was used for conjugated enzyme concentration measurements. Enzyme concentration is expressed in milligrams per milliliter.

3. RESULTS

3.1 BCA Protein Data

Enzyme conjugated to the silica particles was measured as protein using the BCA protein method. Optical density (OD) was first calibrated against known protein concentrations, and a regression was calculated. The enzyme-conjugated particle sample was then measured to determine protein concentration. The BCA calibration data are presented in Figure 4 below.

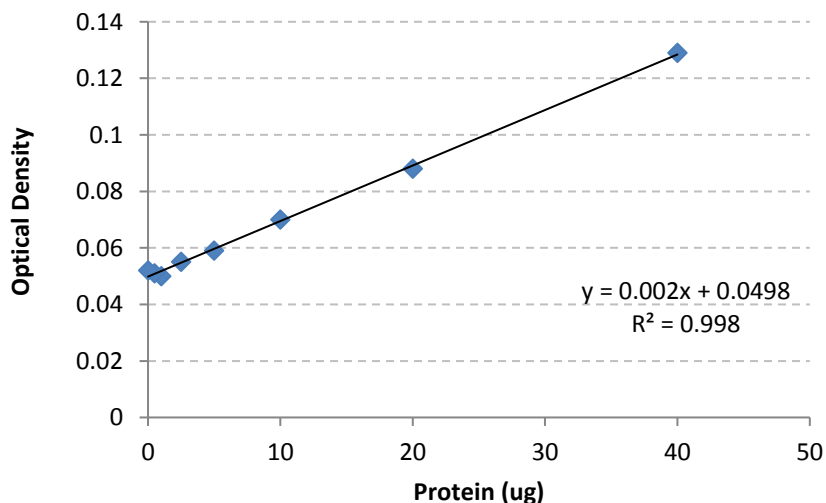


Figure 4. Graphed results from BCA calibration assay for eventual use in determining particle-binding efficiency and enzymatic activity.

In the conjugation methods described above, 1 mg of silica particles was combined with 50 μg of the OPAA mutant Y212F. The conjugated enzyme was washed multiple times to remove unbound enzyme. Enzyme-conjugated particle samples had an OD of 0.1175 for an average protein concentration of 33.85 $\mu\text{g}/\text{mL}$. The protein yield that was calculated from the BCA resulted in a protein yield of 67.7% of the initial amount.

Assuming all particles are spheres, and the density of silica is 2 mg/cm^3 , the mass of a single 80 nm diameter silica particle can be calculated, yielding a total of 1.865×10^{12} particles in 1 mg of silica. Given a molecular weight for the enzyme of 50 kDa and a mass of $33.85 \text{ } \mu\text{g}$ of enzyme per mg of silica, there are 4.06×10^{14} molecules of enzyme per mg of silica. This corresponds to 217 molecules of enzyme per 80 nm silica particle. If we assume that each enzyme is a sphere (potentially a gross simplification), the enzyme takes up a footprint on the surface of the particle related to the cross-sectional area of the circle defined by the radius of the sphere. For a spherical 50 kDa protein, the radius is thought to be 2.4 nm.¹ Thus, the area of the surface occupied by each enzyme is 18.1 nm^2 . Therefore, the 217 enzyme molecules per particle will cover 3928 nm^2 . Each particle has a surface area of $20,106 \text{ nm}^2$, which means that the percentage of enzyme-covered surface is around 19.5%.

3.2 Results on DFP

Fluoride release from the substrate was plotted versus time. The time points selected for measuring specific activity should be obtained from a linear portion of any curve. This is usually within the first 3 min of data collection. Fluoride concentration curves from the DFP assay are presented in Figure 5.

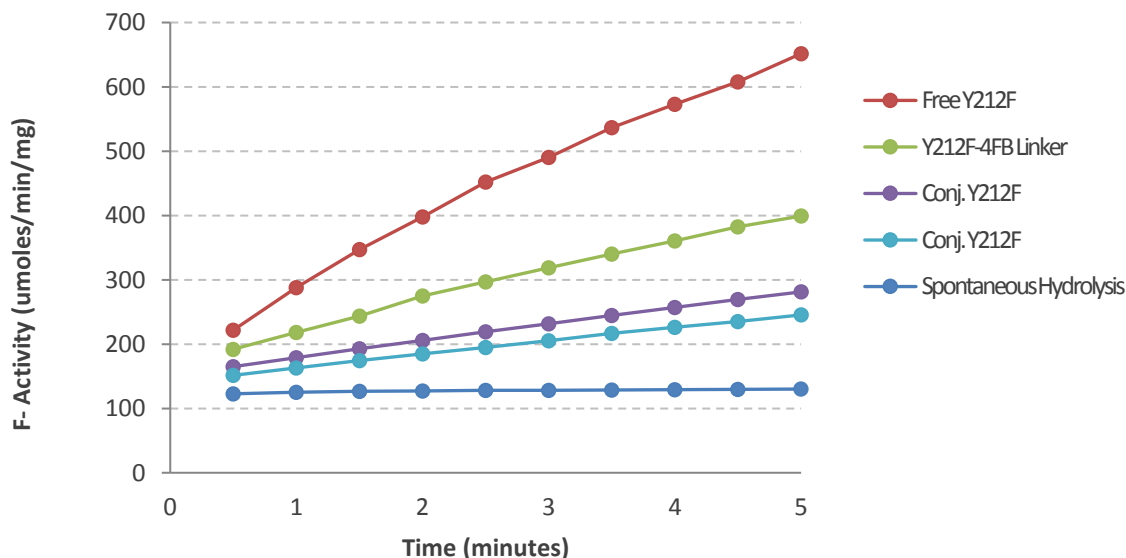


Figure 5. Fluoride assay results from three forms of the Y212F enzyme on DFP.

The results of fluoride ion probe testing on DFP, conducted by nanoComposix personnel, are shown in Table 1. Data points used for specific activity calculations were collected as soon as possible after enzyme addition and once the fluoride release had reached a linear response.

¹ Erickson, H.P. Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biol. Proced. Online* **2009**, *11*, 32–51.

Table 1. Results of Fluoride Release Assay from DFP Testing of Y212F Enzyme, before and after Conjugation to SiO₂ Particles

Substrate	DFP	DFP	DFP	DFP	DFP
Enzyme	No Enzyme	Free Y212F	Y212F-4FB Linker	Conj. Y212F	Conj. Y212F
μmol/min/mg	–	143.059	87.818	26.781	26.948
μmoles F-/min	0.002	0.251	0.154	0.063	0.050
μM F-/min	0.988	100.256	61.448	25.383	19.959
Enz start (mg/mL)	23.36	0.2336	0.714	0.03385	0.03385
Sub start (μmol/μL)	0.300	0.300	0.300	0.300	0.300
Enz (mg)	–	0.00175	0.00175	0.00237	0.00185
Enz/Particle (μL)	–	7.5	2.45	70	54.7
μL 0.3 M Sub	25	25	25	25	25
μM [substrate]	3000	3000	3000	3000	3000
Total assay vol (mL)	2.5	2.5	2.5	2.5	2.5
Spont t=1		126.65	126.65	126.65	126.65
Spont t=2		127.15	127.15	127.15	127.15
Enz t=1	126.65	346.97	243.51	192.81	174.26
Enz t=2	127.15	397.59	274.73	205.99	184.73
% [sub] hydrolyzed	4.24	13.25	9.16	6.87	6.16
BTP (μL)	2450.00	2442.50	2447.55	2380.00	2395.30
Time (min)	Spontaneous Hydrolysis	Free Y212F	Y212F-4FB Linker	Conj. Y212F	Conj. Y212F
0.5	122.77	221.80	192.06	165.02	151.48
1.0	125.18	287.86	218.37	179.07	163.10
1.5	126.65	346.97	243.51	192.81	174.26
2.0	127.15	397.59	274.73	205.99	184.73
2.5	128.14	452.07	296.96	219.22	195.07
3.0	128.14	490.56	318.51	231.50	205.19
3.5	128.64	536.49	340.29	244.46	216.68
4.0	129.14	573.17	360.74	257.14	226.16
4.5	129.65	607.62	382.42	269.43	235.13
5.0	130.15	651.70	399.14	281.22	245.41

F-: fluoride ion; Conj.: conjugated; –: no data; Enz: enzyme; Sub: substrate; vol: volume; Spont: spontaneous; and t: time.

Table 2. Summary Results from Testing on DFP Substrate at nanoComposix

Parameter	Free Enzyme	Enzyme plus 4FB Linker	Conj. Y212F
Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	143.06	87.82	26.78
	–	–	26.95
Specific activity average ($\mu\text{mol}/\text{min}/\text{mg}$)	–	–	26.86
DFP specific activity retained (%)	–	–	18.78

Conj: conjugated; –: no data.

3.3 Results on GD

Changes in fluoride concentration during the enzyme assays on GD are represented in Figure 6.

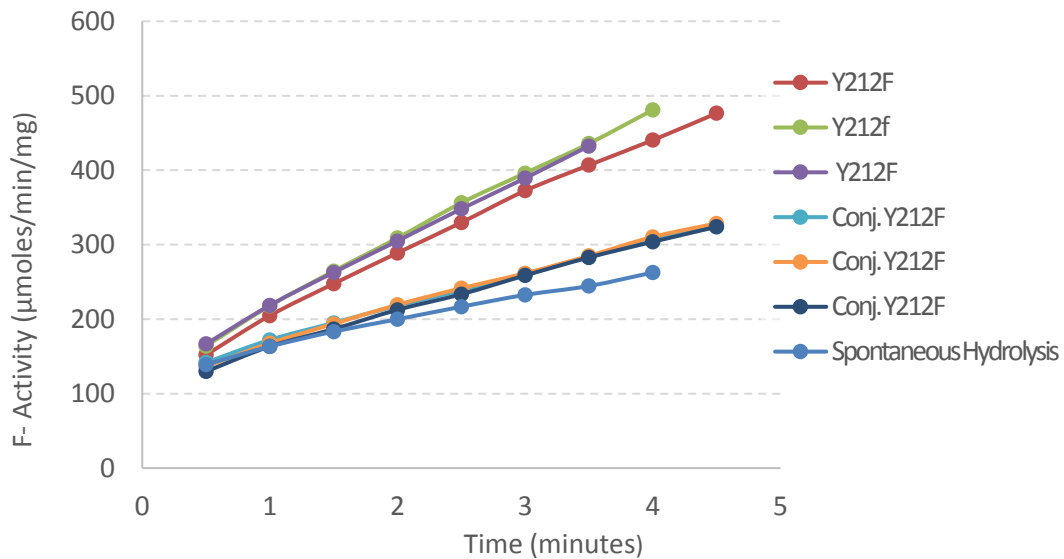


Figure 6. Graphical presentation of Y212F activity on GD.

The results of fluoride ion probe testing of conjugated enzyme on GD, conducted at ECBC, are displayed in Table 3. A summary of the results of specific activities on GD, which were retained after conjugation to SiO_2 particles, are listed in Table 4.

Table 3. Results of Fluoride Release Assay from GD Testing of Y212F Enzyme before and after Conjugation to SiO₂ Particles

Substrate	GD	GD	GD	GD	GD	GD
Enzyme	Y212F	Y212F	Y212F	Conj. Y212F	Conj. Y212F	Conj. Y212F
μmol/min/mg	284.67	317.85	298.59	41.36	46.00	40.99
μmoles F ⁻ /min	0.133	0.149	0.140	0.042	0.055	0.056
μM F ⁻ /min	53.200	59.400	55.800	16.800	21.800	22.200
Enz start (mg/mL)	0.2336	0.2336	0.2336	0.03385	0.03385	0.03385
Sub start (μmol/μL)	0.300	0.300	0.300	0.300	0.300	0.300
Enz (mg)	0.00047	0.00047	0.00047	0.00102	0.00118	0.00135
Enz/Particle (μL)	2	2	2	30	35	40
μL 0.3 M Sub	25	25	25	25	25	25
μM [substrate]	3000	3000	3000	3000	3000	3000
Total assay vol (mL)	2.5	2.5	2.5	2.5	2.5	2.5
Spont t=1	199.00	199.00	161.40	199.00	199.00	199.00
Spont t=2	213.80	213.80	175.70	213.80	213.80	213.80
Enz t=1	247.60	264.50	262.80	195.00	193.70	186.70
Enz t=2	289.00	309.00	305.00	218.20	219.40	212.60
% [sub] hydrolyzed	9.633	10.300	10.167	7.273	7.313	7.087
BTP (μL)	2448.00	2448.00	2448.00	2420.00	2415.00	2410.00
Time (min)	Y212F	Y212F	Y212F	Conj. Y212F	Conj. Y212F	Conj. Y212F
0.5	152.10	164.00	166.70	141.90	136.40	129.80
1.0	204.80	218.00	218.60	172.20	167.70	163.40
1.5	247.60	264.50	262.80	195.00	193.70	186.70
2.0	289.00	309.00	305.00	218.20	219.40	212.60
2.5	329.80	356.20	348.10	238.80	241.50	233.50
3.0	372.50	396.10	389.30	261.10	261.60	258.70
3.5	406.90	435.90	432.60	283.40	285.00	282.90
4.0	440.40	481.00		306.40	310.40	303.90
4.5	476.50			324.30	328.50	324.00

F⁻: fluoride ion; Conj.: conjugated; -: no data; Enz: enzyme; Sub: substrate; vol: volume; Spont: spontaneous; and t: time.

Table 4. Summary of Retained Specific Activity of Enzyme Y212F on GD

Parameter	Y212F	Conj. Y212F
Specific activity (μmol/min/mg)	284.67	41.36
	317.85	46.00
	298.59	40.99
Specific activity average (μmol/min/mg)	300.37	42.78
GD specific activity retained (%)	NA	14.24

4. SUMMARY

Immobilizing an enzyme on a particle is a two-step process. The first step is to hybridize a linker to the enzyme, then conjugate the linker to the silica particle. The enzyme activity was measured by nanoComposix personnel on DFP attachment was started and after each step in the process. After completion, the immobilized enzyme activity was measured at ECBC using GD as a substrate.

In initial testing by nanoComposix personnel using DFP as a substrate, it was observed that when an initial short-linker was used, the result was very low retained activity, even after only the linker hybridization step. A longer linker was subsequently used, which resulted in better activity.

Data described herein indicates that although a significant percentage (67.7%) of the enzyme was successfully conjugated to the silica particles, only 14.24% of the activity on GD was retained. A loss in activity (38.6% loss) was observed at the 4FB linker hybridization step, and an additional loss occurred after the silica particle-conjugation step. Therefore, a large portion of the enzymatic activity was lost through conjugation to the particles. Ideally, there is a point of optimal protein-loading and linker length that can maintain activity. These must be measured against stability, shelf life, logistical burden, and effectiveness in a delivery system. More of these points need to be addressed as therapeutic development continues.

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ACRONYMS AND ABBREVIATIONS

BCA	bicinchoninic acid
BTP	bis-trispropane
C6-S-4FB	C6-succinimidyl 4-formylbenzoate
DFP	diisopropyl fluorophosphate
ECBC	U.S. Army Edgewood Chemical Biological Center
GD	pinacolyl methyl phosphonofluoridate, soman
OD	optical density
OPAA	organophosphorus acid anhydrolase
S-4FB	succinimidyl 4-formylbenzoate
S-HyNic	succinimidyl 6-hydrazinonicotinate acetone hydrazone
TEM	transmission electron microscope

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