

Supporting Information

Evaluation of Thiol Raman Activities and pKa Values using Internally-referenced Raman-based pH Titration

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S1. Derivation of Eq. 5 in the manuscript

$$pH - pK_a = \log \frac{R^0 - R^{pH}}{R^{pH}} \quad \text{Eq. S1 (Eq. 4 in manuscript)}$$

By rearranging Eq. S1,

$$10^{(pH-pK_a)} = \frac{R^0 - R^{pH}}{R^{pH}} \quad \text{Eq. S2}$$

By simplifying Eq. S2,

$$10^{(pH-pK_a)} = \frac{R^0}{R^{pH}} - 1 \quad \text{Eq. S3}$$

By further rearranging Eq. S3,

$$R^{pH} = \frac{R^0}{1 + 10^{(pH-pK_a)}} \quad \text{Eq. S4}$$

$$R^{pH} = \frac{R^0}{1 + e^{2.303(pH-pK_a)}} \quad \text{Eq. S5 (Eq. 5 in manuscript)}$$

S2. Derivation of Eq. 9 in the manuscript

Using the standard Henderson-Hasselbatch equation for conversion of dithiol to mono protonated dithiol.

$$pH = pK_{a1} + \log \frac{[RS_2H^-]_{pH}}{[RS_2H_2]_{pH}} \quad \text{Eq. S6}$$

Using the standard Henderson-Hasselbatch equation for conversion of mono protonated dithiol to fully protonated dithiol.

$$pH = pK_{a2} + \log \frac{[RS_2^{2-}]_{pH}}{[RS_2H^-]_{pH}} \quad \text{Eq. S7}$$

By adding Eq. S6 and Eq. S7,

$$2pH = pK_{a1} + pK_{a2} + \log \frac{[RS_2^{2-}]_{pH}}{[RS_2H_2]_{pH}} \quad \text{Eq. S8}$$

The concentration of intact dithiol $[RS_2H_2]_0$ is equal to the sum of non-ionized dithiol concentration $[RS_2H_2]_{pH}$ and concentration of mono thiolated dithiol $[RS_2H^-]_{pH}$ and fully protonated dithiol $[RS_2^{2-}]_{pH}$ at specified pH conditions.

$$[RS_2H_2]_0 = [RS_2H_2]_{pH} + [RS_2H^-]_{pH} + [RS_2^{2-}]_{pH} \quad \text{Eq. S9}$$

By rearranging Eq. S9,

$$[RS_2^{2-}]_{pH} = [RS_2H_2]_0 - [RS_2H_2]_{pH} - [RS_2H^-]_{pH} \quad \text{Eq. S10}$$

By substituting $[RS_2^{2-}]_{pH}$ (Eq. S10) in Eq. S8,

$$2pH = pK + \log \frac{[RS_2H_2]_0 - [RS_2H_2]_{pH} - [RS_2H^-]_{pH}}{[RS_2H_2]_{pH}} \quad \text{Eq. S11}$$

Where,

$$pK = pK_{a1} + pK_{a2} \quad \text{Eq. S12}$$

$$R^0 = \frac{I_{SH}^0}{I_{ref}^0} = \frac{[RS_2H_2]_0 \sigma_{S_2H_2}}{[REF]_0 \sigma_{ref}} \quad \text{Eq. S13}$$

By rearranging Eq. S10,

$$[RS_2H_2]_0 = \frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2}} R^0 \quad \text{Eq. S14}$$

By rearranging Eq. 6,

$$[RS_2H^-]_{pH} = [RS_2H_2]_{pH} \cdot 10^{pH - pK_{a1}} \quad \text{Eq. S15}$$

$$R^{pH} = \frac{I_{SH}^{pH}}{I_{ref}^{pH}} = \frac{[RS_2H_2]_{pH} \sigma_{S_2H_2} + [RS_2H^-]_{pH} \sigma_{S_2H^-}}{[REF]_0 \sigma_{ref}} \quad \text{Eq. S16}$$

By substituting $[RS_2H^-]_{pH}$ (Eq. S15) in Eq. S16,

$$R^{pH} = \frac{[RS_2H_2]_{pH} \sigma_{S_2H_2} + ([RS_2H_2]_{pH} \cdot 10^{pH - pK_{a1}}) \sigma_{S_2H^-}}{[REF]_0 \sigma_{ref}} \quad \text{Eq. S17}$$

By rearranging Eq. S17,

$$[RS_2H_2]_{pH} = \frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} \cdot 10^{pH - pK_{a1}}} R^{pH} \quad \text{Eq. S18}$$

By substituting $[RS_2H_2]_0$ (Eq. S14) and $[RS_2H^-]_{pH}$ (Eq. S15) in Eq. S11,

$$2pH = pK + \log \frac{\frac{[REF]_0 \sigma_{ref} R^0}{\sigma_{S_2H_2}} - [RS_2H_2]_{pH} - [RS_2H_2]_{pH} \cdot 10^{pH - pK_{a1}}}{[RS_2H_2]_{pH}} \quad \text{Eq. S19}$$

Simplifying Eq. S19 into Eq. S20.

$$2pH = pK + \log \frac{\frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2}} R^0 - [RS_2H_2]_{pH} (1 + 10^{pH - pK_{a1}})}{[RS_2H_2]_{pH}} \quad \text{Eq. S20}$$

Substituting $[RS_2H_2]_{pH}$ (Eq. S18) in Eq. S20 leads to Eq. S21,

$$2pH = pK + \log \frac{\frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2}} R^0 - \frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}} R^{pH} (1 + 10^{pH-pK_{a1}})}{\frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}} R^{pH}} \quad \text{Eq. S21}$$

By multiplying both numerator and denominator of log term by $\frac{\sigma_{S_2H_2}}{[REF]_0 \sigma_{ref}}$,

$$(2pH = pK + \log \frac{[\frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2}} R^0 - \frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}} R^{pH} (1 + 10^{pH-pK_{a1}})] \frac{\sigma_{S_2H_2}}{[REF]_0 \sigma_{ref}}}{[\frac{[REF]_0 \sigma_{ref}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}} R^{pH}] \frac{\sigma_{S_2H_2}}{[REF]_0 \sigma_{ref}}})$$

$$2pH = pK + \log \frac{R^0 - \frac{\sigma_{S_2H_2} R^{pH} (1 + 10^{pH-pK_{a1}})}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}}}{\frac{\sigma_{S_2H_2}}{\sigma_{S_2H_2} + \sigma_{S_2H^-} x 10^{pH-pK_{a1}}} R^{pH}} \quad \text{Eq. S22}$$

By multiplying both numerator and denominator of log term by $(1/\sigma_{S_2H_2})$,

$$2pH = pK + \log \frac{R^0 - \frac{R^{pH} (1 + 10^{pH-pK_{a1}})}{1 + (\sigma_{S_2H^-} / \sigma_{S_2H_2}) 10^{pH-pK_{a1}}}}{\frac{R^{pH}}{1 + (\sigma_{S_2H^-} / \sigma_{S_2H_2}) \cdot 10^{pH-pK_{a1}}}} \quad \text{Eq. S23}$$

But,

$$\sigma_{S_2H^-} / \sigma_{S_2H_2} = \alpha \quad \text{Eq. S24}$$

$$2pH = pK + \log \frac{R^0 - \frac{R^{pH} (1 + 10^{pH-pK_{a1}})}{1 + \alpha 10^{pH-pK_{a1}}}}{\frac{R^{pH}}{1 + \alpha \cdot 10^{pH-pK_{a1}}}} \quad \text{Eq. S25}$$

By multiplying both numerator and denominator of log term by $1 + \alpha 10^{pH-pK_{a1}}$,

$$2pH = pK + \log \frac{R^0 (1 + \alpha 10^{pH-pK_{a1}}) - R^{pH} (1 + 10^{pH-pK_{a1}})}{R^{pH}} \quad \text{Eq. S26}$$

$$2pH = pK + \log \frac{R^0 + R^0 \alpha 10^{pH-pK_{a1}} - R^{pH} - R^{pH} 10^{pH-pK_{a1}}}{R^{pH}} \quad \text{Eq. S27}$$

By rearranging Eq.S27,

$$10^{(2pH-pK)} = \frac{R^0 + \alpha R^0 10^{(pH-pK_{a1})}}{R^{pH}} - (1 + 10^{(pH-pK_{a1})}) \quad \text{Eq. S28}$$

$$R^{pH} = \frac{R^0 + \alpha R^0 10^{(pH-pK_{a1})}}{10^{(2pH-pK)} + (1 + 10^{(pH-pK_{a1})})} \quad \text{Eq. S29}$$

$$R^{pH} = \frac{R^0 + \alpha R^0 10^{(pH-pK_{a1})}}{10^{(2pH-pK_{a1}-pK_{a2})} + (1 + 10^{(pH-pK_{a1})})} \quad \text{Eq. S30}$$

By Simplifying Eq. S30,

$$R^{pH} = \frac{R^0 + \alpha R^0 10^{(pH-pK_{a1})}}{1 + 10^{(pH-pK_{a1})} (1 + 10^{(pH-pK_{a2})})} \quad \text{Eq. S31}$$

S3. R code for least square curve-fitting with Eq. 9

```
#####fr is the least square function that need to be optimized#####
fr=function(par,ph,Rph){
  R0=par[1]
  alpha=par[2]
  pka1=par[3]
  pka2=par[4]
  sum(((R0+alpha*R0*10^(ph-pka1))/(1+10^(ph-pka1)*(1+10^(ph-pka2))))-Rph)^2)
}
#####
#####
# pka.fit is the function to fit the parameters
#
# par is a vector of the initial guess for the parameters R0,alpha, pka1 and pka2
#
# ph is the experimental value for ph
#
# Rph is the experimental value for Rph
#
# Returning values are fitted parameters, fitted Rph and MSE
#
#####
#####

pka.fit=function(par,ph,Rph){
  res=optim(par, fr, ph=ph, Rph=Rph)
  R0.f=res$par[1]
  alpha.f=res$par[2]
  pka1.f=res$par[3]
  pka2.f=res$par[4]
  Rph.f=(R0.f+alpha.f*R0.f*10^(ph-pka1.f))/(1+10^(ph-pka1.f)*(1+10^(ph-pka2.f)))
  MSE=sum((Rph.f-Rph)^2)
  return(list(R0.f=R0.f,alpha.f=alpha.f,pka1.f=pka1.f, pka2.f=pka2.f, Rph.f=Rph.f, MSE=MSE))
}
```


S4. Raman-based pH titration for the thiol pK_a determination for Glutathione

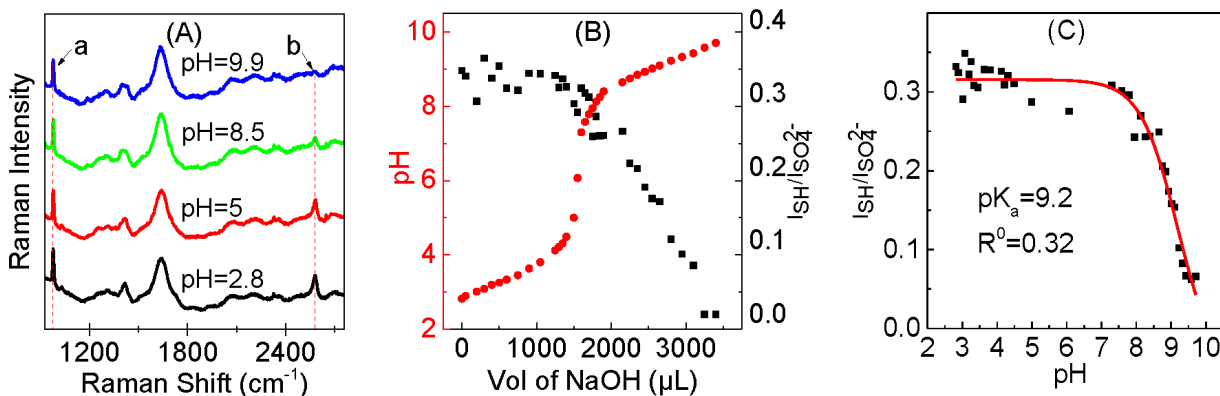


Figure S1: Internally-referenced Raman pH titration for the determination of thiol pK_a values in GSH. (A) Representative Raman spectra of GSH as a function of solution pH ((a) SO₄²⁻, (b) SH peak). The spectra are normalized by the sulfate peak intensity. (B) Solution pH (red circles, left scale) and I_{SH}/I_{SO₄²⁻} (black squares, right scale) as a function of titrant (500 mM NaOH) volume. (C) The relative S-H peak intensity as a function of solution pH. The solid curves are by fitting the experimental data with the Boltzmann function fitting using Origin Pro. The GSH and Na₂SO₄ concentrations in the initial titration solution are 0.22 M and 0.15 M, respectively.

S5. Raman-based pH titration for the thiol pK_a determination for 2-Mercaptoethanol

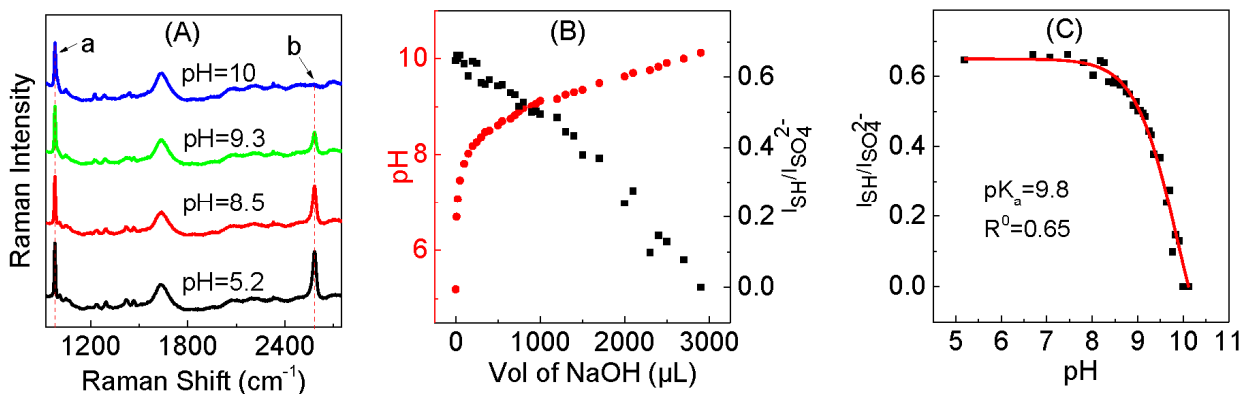


Figure S2: Internally-referenced Raman pH titration for the determination of thiol pK_a values in 2-ME. (A) Representative Raman spectra of 2-ME as a function of solution pH ((a) SO₄²⁻, (b) SH peak). The spectra are normalized by the sulfate peak intensity. (B) Solution pH (red circles, left scale) and $I_{\text{S-H}}/I_{\text{SO}_4^{2-}}$ (black squares, right scale) as a function of titrant (500 mM NaOH) volume. (C) The relative S-H peak intensity as a function of solution pH. The solid curves are by fitting the experimental data with the Boltzmann function fitting using Origin Pro. The 2-ME and Na₂SO₄ concentrations in the initial titration solution are 0.42 M and 0.15 M, respectively.

S6. Raman-based pH titration for the thiol pK_a determination for Cysteamine

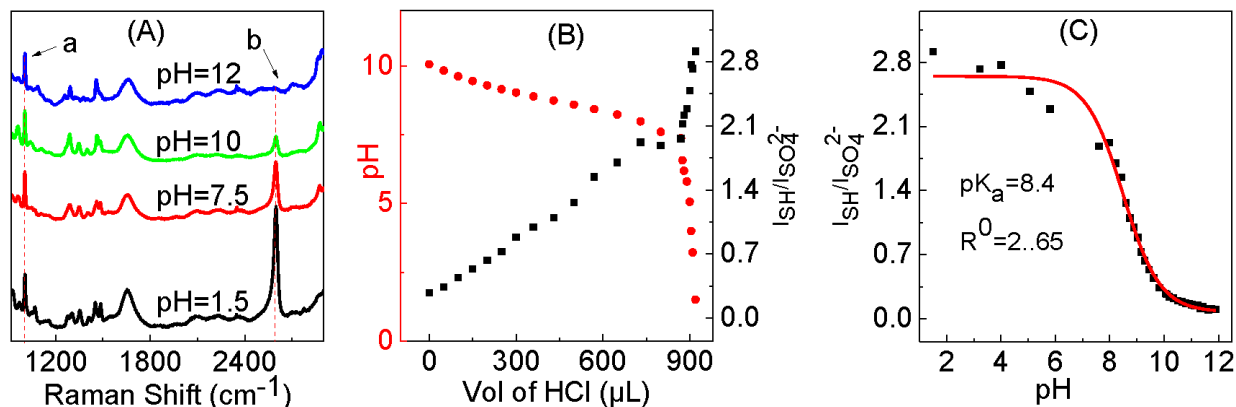


Figure S3: Internally-referenced Raman pH titration for determination of the thiol pK_a values in CyA. (A) Representative Raman spectra of CyA as a function of solution pH. (a. SO₄²⁻, b.SH peak) The spectra are normalized by the sulfate peak intensity. (B) Solution pH (red circles, left scale) and I_{S-H}/I_{SO₄²⁻} (black squares, right scale) as a function of titrant (500 mM NaOH) volume. (C) The relative S-H peak intensity as a function of solution pH. The solid curves are by fitting the experimental data with the Boltzmann function fitting using Origin Pro. The CyA and Na₂SO₄ concentrations in the initial titration solution are 2.5 M and 0.15 M, respectively.

S7. Comparison of the thiol pKa determination for DTT using the peak intensity ratio and integrated peak area ratio.

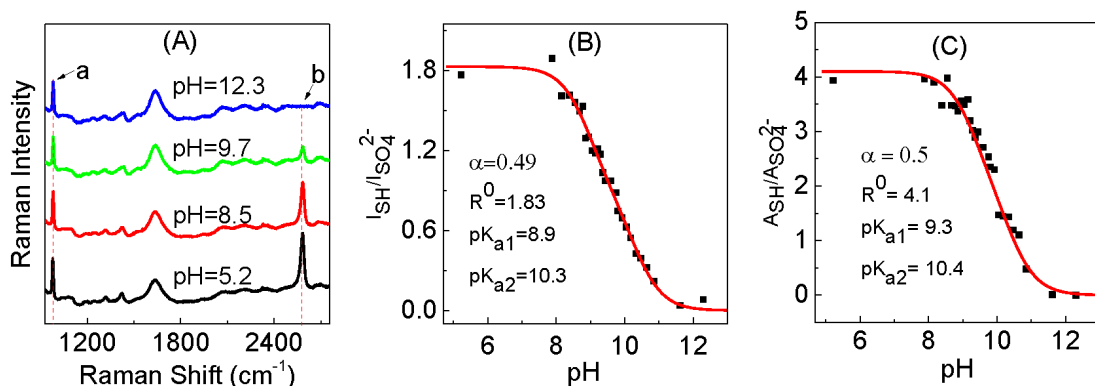


Figure S4: (A) Representative Raman spectra of DTT as a function of solution pH. ((a) SO_4^{2-} , (b) S-H peak) The spectra are normalized by the sulfate peak intensity. (B) the S-H/ SO_4^{2-} peak intensity ratio as a function of solution pH. (C) the S-H/ SO_4^{2-} integrated peak intensity ratio as a function of solution pH. The solid curve in (B) and (C) are obtained by least-square curve-fitting the experimental data with the Eq. 9 using the R program shown in the supporting information. The DTT thiol pK_a values determined with the intensity ratio are very similar to those obtained with curve-fitting the integrated peak area ratios.