Bioconjugate Chemistry

Article

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General Chemoselective and Redox-Responsive Ligation and Release Strategy

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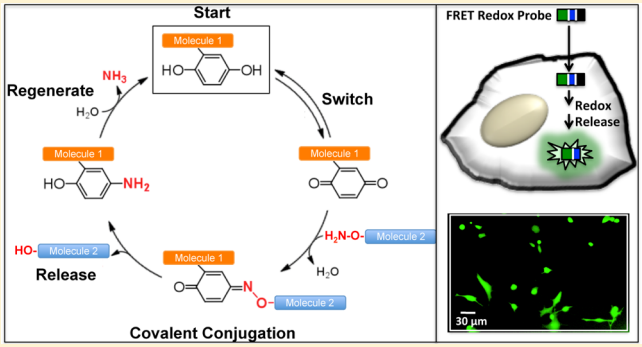
Supporting Information

ABSTRACT: We report a switchable redox click and cleave reaction strategy for conjugating and releasing a range of molecules on demand. This chemoselective redox-responsive ligation (CRRL) and release strategy is based on a redox switchable oxime linkage that is controlled by mild chemical or electrochemical redox signals and can be performed at physiological conditions without the use of a catalyst. Both conjugation and release reactions are kinetically well behaved and quantitative. The CRRL strategy is synthetically modular and easily monitored and characterized by routine analytical techniques. We demonstrate how the CRRL strategy can be used for the dynamic generation of cyclic peptides and the ligation of two different peptides that are stable but can be selectively cleaved upon changes in the redox environment. We also demonstrate a new redox based delivery of cargoes to live cells strategy via the CRRL methodology by synthesizing a FRET redox-responsive probe that is selectively activated within a cellular environment. We believe the ease of the CRRL strategy should find wide use in a range of applications in biology, tissue engineering, nanoscience, synthetic chemistry, and material science and will expand the suite of current conjugation and release strategies.



INTRODUCTION

The ability to chemoselectively ligate a range of biomolecules, polymers, nanoparticles, and nanomaterials is fundamental to current chemistry research and is crucial for a wide range of applications from protein conjugation, pharmaceutical therapies, molecular electronics, polymer synthesis, drug delivery, and tissue engineering.[1] The most used methods rely on click chemistry type reactions that span Diels–Alder, Michael additions, thiol–ene reactions, Staudinger reactions, nucleophilic ring-opening reactions, 1,3-dipolar cycloaddition, oxime/hydrazone formation reactions, and native chemical ligations.[2] Although several conjugation methods exist and have been used for many applications, the use of these methods for *in vitro*/*in vivo* biological studies and applications has been severely limited due to the special nature of the biological environment that limits the scope of reactions that may be employed.[3] This subset of conjugation reactions for *in vitro*/*in vivo* studies and applications ideally, at physiological conditions, require kinetically well-behaved reactions that are bio-orthogonal, stable (covalent bonds), and modular to conjugate a diverse range of molecules and noncytotoxic compounds. Moreover, a conjugation strategy that can be (1) monitored and characterized with simple nonintrusive analytical methods, and (2) turned on or off for conjugation, and can (3) release molecules and be inexpensive would complement and expand the scope of existing conjugation/release methods for a range of new applications.

It is well known that aminooxy groups (R-ONH$_2$) selectively react with carbonyl groups to form stable oxime products, which have been used as a popular bioconjugation methodology for the preparation of ligand microarrays and cell arrays,[4−6] therapeutics,[7] fluorescent labeling,[8] combinatorial libraries,[9] artificial proteins,[10] glycoprotein mimetics,[11] chemically tunable polymers,[12] and biosensors.[13] Oxime formation is chemoselective and stable at physiological conditions.[14−16] There are many types of oximes reported and characterized, and each have unique characteristics including the quinone oxime; having an oxime moiety ($R_1R_2C{=}N\text{-}O\text{-}R_3$) directly adjacent to the aromatic ring.[17] Among them, the quinone oxime ether (QO) and ester have been studied in the field of stereoelectronic control in organic chemistry[18] and applied to cytotoxin molecules,[19] therapeutics,[20] and photoinduced iminyl radical generation.[21] We have extensively studied and employed the quinone oxime ether (QO) as a quantitative immobilization strategy on gold and indium tin oxide (ITO) surfaces for a range of biotechnological

Received: December 11, 2013
Revised: February 21, 2014
Published: February 24, 2014

   dx.doi.org/10.1021/bc400565y | *Bioconjugate Chem.* 2014, 25, 543−551

and cell behavior studies.[22−29] However, until now, the QO with its interesting aromatic structure and redox properties has not been explored as a solution based conjugation or cleavage strategy.

The ability to cleave specific chemical linkages with mild stimuli is a powerful tool in designing small molecule drugs, *in vivo* biosensors, drug delivery methods, solid phase synthesis, and protein−protein interactions.[30−32] There are several popular classes of cleavable linkers for these applications: cleavage by reduction (sulfhydryl and diazobenzene), oxidation (periodate), nucleophilic substitution, and base labile sulfones.[33] Among them, reduction induced cleavage is attractive due to its relatively mild induction which can be performed at physiological conditions. For example, *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and succinimidyl oxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene (SMPT) are often used for the preparation of immunotoxin conjugates that contain a monoclonal antibody which is cross-linked to a protein toxin molecules, the antibody directed against tumor-associated antigens. Lambert et al. showed that a disulfide linkage between the antibody and toxin molecules results in a potent toxicity when compared to that of noncleavable linkages.[34]

In this article, we develop a general methodology termed chemoselective redox-responsive ligation (CRRL) and release strategy for molecular conjugation and cleavage of a range of molecules in solution. This methodology relies on the redox controlled activation, conjugation, release, and regeneration of a series of electroactive hydroquinone derived molecules. The strategy is based on the reaction of aminooxy-tethered ligands with a redox controlled benzoquionone group for subsequent oxime formation and release. We believe, this CRRL strategy is dynamic, switchable, kinetically well behaved, stable, inexpensive, synthetically flexible, and can be regenerated for subsequent rounds of conjugation and release. Furthermore, the CRRL reactions can be monitored by simple UV−vis spectroscopy methods and controlled by electrochemistry (either chemical redox reagents or applied potentials). We characterize the CRRL chemical sequence and then demonstrate the utility of the CRRL strategy in two ways: (1) by generating dynamic peptides for cell inhibition assays and (2) as a redox based system to deliver cargoes specifically to the interior of cells.

MATERIALS AND METHODS

Spectroscopy. Spectra were obtained using a diode-array spectrophotometer for both standard and kinetics measurements. All of the measurements were performed using quartz spectrophotometer cells in Dulbecco's PBS (used as received) at room temperature. All measurements were initiated immediately after sample preparation.

Chromatography and Mass Spectrometry. Analytical HPLC was performed on a diode array detector and an automated sampler. The samples were eluted through an Atlantis analytical column (C18, 5 μm, 6 × 150 mm) with a binary solvent system (solvent A, optima grade water with 5% optima grade acetonitrile and 0.1% TFA; solvent B, acetonitrile with 5% water and 0.1% TFA) with a gradient of 0−100% solvent B over 50 min (1 mL/min). For the LC-MS data for intra-CRRL RGD (**6**) and inter-CRRL RGD (**9**) peptides, samples were run on a UPLC system coupled to a mass spectrometer (scanning 300−1600 m/z) using a UPLC column (BEH C18 1.7 μm, 2.1 × 150 mm, 30 °C). The elution occurred in a gradient of binary solvents (solvent A, optima grade water with 0.2% formic acid; solvent B, Optima grade acetonitrile with 0.2% formic acid), where solvent B increased from 1% to 40% over 20 min.

CRRL Activation, Conjugation, Release, and Regeneration. Activation: the hydroquinone (HQ) can be oxidized to the benzoquinone (BQ) via chemical or electrochemical methods. Addition of Cu^{2+} (2 equivalents) in pH 7.4 PBS instantly oxidizes HQ to BQ quantitatively. The HQ can also be oxidized to BQ in solution via electrochemical methods (potentiostat) by application of an oxidative potential (+750 mV, 1 min, versus a Ag/AgCl reference electrode) to a stirring solution of HQ (0.1 mM, pH 7.4, PBS) in an electrochemical cell containing a gold working electrode. Conjugation: the benzoquinone (BQ) can form a stable quinone oxime ether (QO) when reacted with aminooxy-containing ligands (R-ONH_2) in a range of pH conditions (pH 1.0 to 10). Release: the quinone oxime ether (QO) can be chemically or electrochemically cleaved to release the aminooxy ligand (R-ONH_2) as a primary alcohol (R-OH) with the generation of aminophenol (AP). Addition of either a soluble reducing agent (DTT, 5 mM, pH 7.4, PBS, 10 h) or electrochemical reduction (−100 mV, 1 min, pH 7.4, PBS) causes the efficient cleavage of QO to AP with high yield (>95%). Regeneration: the aminophenol (AP) can be oxidized to the quinoneimine, which rapidly hydrolyzes to generate the benzoquinone (BQ) with the release of ammonia. The BQ is then reduced to generate the HQ via a classic electrode chemical (EC) process.[35]

Fibroblast (Fb) Culture. Swiss 3T3 albino mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% calf bovine serum (CBS) and 1% penicillin/streptomycin at 37 °C in 5% CO_2.

Cell Adhesion Inhibition Assay. 3T3 Swiss Albino mouse fibroblasts were pretreated with peptides (**6**−**11**) at varying concentrations and seeded onto a RGD-presenting SAM gold substrate. We have previously reported and described these types of surfaces for a range of cell behavior studies.[23] Briefly, gold substrates were immersed in a 1 mM ethanolic solution of alkanethiolates (1:99 HQ/TEG) for 12 h. The hydroquinone-containing surfaces were oxidized by applying a potential of 750 mV (vs Ag/AgCl) for 15 s in a 1 M solution of $HClO_4$ using a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN). Then, aminooxy acetic acid-Gly-Arg-Gly-Asp-Ser-amidated was added (10 mM in PBS) for 3 h to install the peptide onto the surface. The fibroblasts were cultured in Dulbecco's modified Eagle's medium with 10% bovine calf serum and 1% penicillin/streptomycin (37 °C in a humidified 5% CO_2 atmosphere). In order to plate cells onto the SAM substrates, cells were removed from the culture flask with a solution of 0.05% trypsin/0.53 mM EDTA and resuspended in serum-free culture medium (5,000−10,000 cells/mL). The 0.3 mL of aliquots are treated with peptides in various concentrations for 5 min and seeded and incubated for 30 min and gently rinsed with PBS. Adhered cells were counted at random locations on the substrates (n = 129). The ratio (%) of adhered cells to the nonpretreated cell control experiments were plotted.

Cell Treatment with Cell-Permeable Redox-Responsive Calcein Dye (CPRRC, 12). Two microliters of 40 mM CPRRC was added to 4 mL of culture media for a final concentration of 10 μM. This mixture was added to fibroblasts in culture for 1 h. The medium was removed, and 4 mL of fresh

dx.doi.org/10.1021/bc400565y | *Bioconjugate Chem.* 2014, 25, 543−551

medium was added. After 1 h, the cells were imaged in PBS with a Nikon TE2000.

Fluorometry. Ten micromolar CPRRC was prepared in 5 mM glutathione at pH 7. To measure the initial fluorescence, the fluorescence was scanned from 515 to 600 nm with 495 nm excitation on a Biocore 765 fluorometer. For the recovery studies, 525 nm was monitored over 3 h every 2 min. After the recovery was complete, 515 to 600 nm was scanned again.

RESULTS AND DISSCUSSION

Figure 1 shows the cartoon scheme and molecular structures of the solution based CRRL conjugation and release strategy. A CRRL receptor can be switched between the off state and on state by mild chemical or electrochemical redox changes. Only when the CRRL receptor is turned on can it react chemoselectively with a CRRL ligand to generate a stable covalent conjugated pair. The covalent ligation product is stable but can be selectively released by a mild chemical or electrochemical reductant. The CRRL cleavage reaction regenerates the receptor group, while the released ligand is irreversibly altered at the ligation site. Subsequent rounds of conjugation and cleavage at physiological conditions (pH 7.4 and 37 °C) can be performed. The reaction is bio-orthogonal, chemoselective, and may be done in complex protein mixtures and cell culture.[22,23]

A hydroquinone (HQ) is the off CRRL receptor and can be converted to the benzoquinone (BQ) (on CRRL receptor) by mild chemical oxidants or electrochemical oxidation. This interconversion process is chemically and electrochemically reversible, which enables switching between the active and inactive forms for chemoselective conjugation. An aminooxy-tethered molecule (R-ONH$_2$; the CRRL ligand) can conjugate selectively to the BQ form generating a covalent and stable quinone oxime ether (QO) as a ligation product. The QO can then be reduced by mild chemical reductants or electrochemical reduction to yield the aminophenol (AP) with release of the ligand as a hydroxyl-terminated molecule (R-OH). The HQ can then be regenerated from aminophenol (AP) by subsequent steps of oxidation, followed by the hydrolysis of imine to generate BQ with the liberation of ammonia and then further reduction of BQ to HQ through a classic EC (electrode and chemical) mechanism.[35]

To monitor and characterize the kinetics of the stable intermediate products of the CRRL conjugation and cleavage strategy, we used standard UV−vis spectroscopy (Figure 2). Upon oxidation of hydroquinone (HQ), benzoquinone (BQ) is formed, which can react with aminooxy groups (R-ONH$_2$) to form the quinone oxime ether (QO). The QO can then be cleaved to generate the aminophenol (AP) with all species having diagnostic absorbances (HQ, 288 nm, ε = 2,520 $M^{-1}cm^{-1}$; BQ, 248 nm, ε = 20,300 $M^{-1}cm^{-1}$; QO, 320 nm, ε = 18,163 $M^{-1}cm^{-1}$; AP, 297 nm, ε = 2,150 $M^{-1}cm^{-1}$; Figure S1, Supporting Information). Figure 2a, left shows the conjugation reaction between BQ and methoxyamine (CH_3ONH_2) characterized by UV−vis spectroscopy. To the solution of BQ (PBS, 200 μM), a solution of methoxyamine (PBS, 100 mM) was added immediately after the initial measurement (0 min). The visible isosbestic point demonstrates that BQ and QO are linearly related by stoichiometry, supporting that the reaction is coupled by the formation of QO and decay of BQ.

The absorbances at 248 and 320 nm were plotted against time in an exponential plot (Figure 2b, left). The exponential graph was fitted to pseudo-first-order reaction kinetics in order

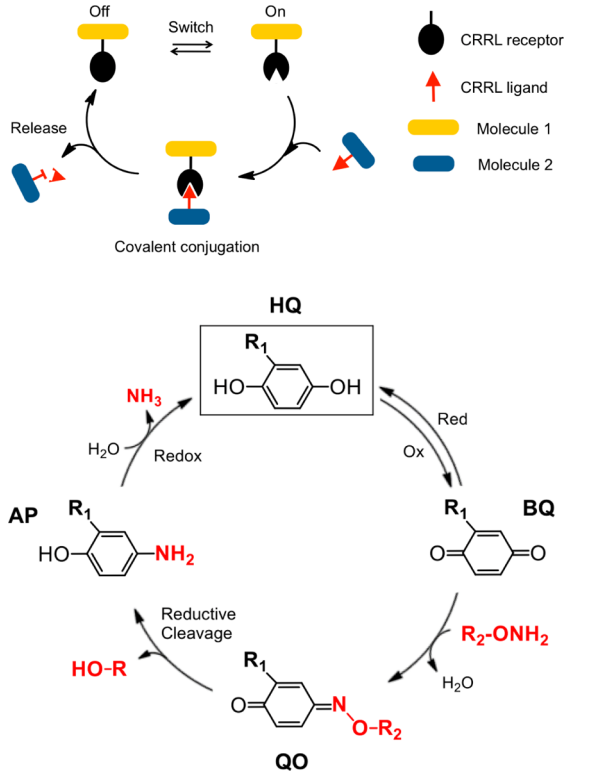


Figure 1. Cartoon scheme and molecular cycle of the chemoselective redox-responsive ligation (CRRL) and release strategy. (Top) A CRRL receptor tethered to molecule 1 can be switched between the "off" state (complete circle) and "on" state (incomplete circle) by mild chemical or electrochemical oxidation and reduction. Only when the receptor is turned "on" can the CRRL ligand (red arrow) react chemoselectively to form a conjugated molecule pair. The covalent ligation product is stable but can be selectively released by a mild chemical or electrochemical reduction. The CRRL cleavage reaction regenerates the receptor group while the ligand is irreversibly altered. Conjugation between the receptor and the ligand, as well as the cleavage reaction, are fast, mild, clean (no need for catalyst), bio-orthogonal, and chemoselective in physiological conditions (pH of 7.4 and 37 °C) and complex protein mixtures (cell lysates), which are essential characteristics for biological applications. (Bottom) A hydroquinone (HQ) is the "off" CRRL receptor and can be converted to the benzoquinone (BQ) ("on" CRRL receptor) by mild chemical or electrochemical oxidation. This activation interconversion process is chemically and electrochemically reversible, which enables switching between the active and inactive forms for chemoselective conjugation. An aminooxy-tethered molecule (R-ONH$_2$; the CRRL ligand) can conjugate selectively to the BQ form generating a covalent and stable quinone oxime ether (QO) as a ligation product. The QO is reduced in mild conditions to yield the aminophenol (AP) with release of the ligand as a hydroxyl-terminated molecule (R−OH). The activation, conjugation, and release steps all occur at physiological conditions (pH 7.4, 37 °C). The HQ can then be regenerated from AP by a subsequent oxidation in low pH followed by hydrolysis of the imine and reduction by a classic electrode−chemical (EC) mechanism.[35]

to obtain k'. After solving for a series of k's from different reaction conditions with excess methoxyamine, the k' values can be plotted against [methoxyamine] (Figure 2c, left) to obtain the second-order rate constant for CRRL conjugation, k = 3.41 × 10^{-2} (±6.5 × 10^{-4}) $M^{-1}s^{-1}$ (PBS, room temperature).

The same experiments and calculations were performed for the CRRL cleavage reaction. To a solution of QO (PBS, 200 μM), dithiothreitol (DTT) (PBS, 100 mM) was added (Figure

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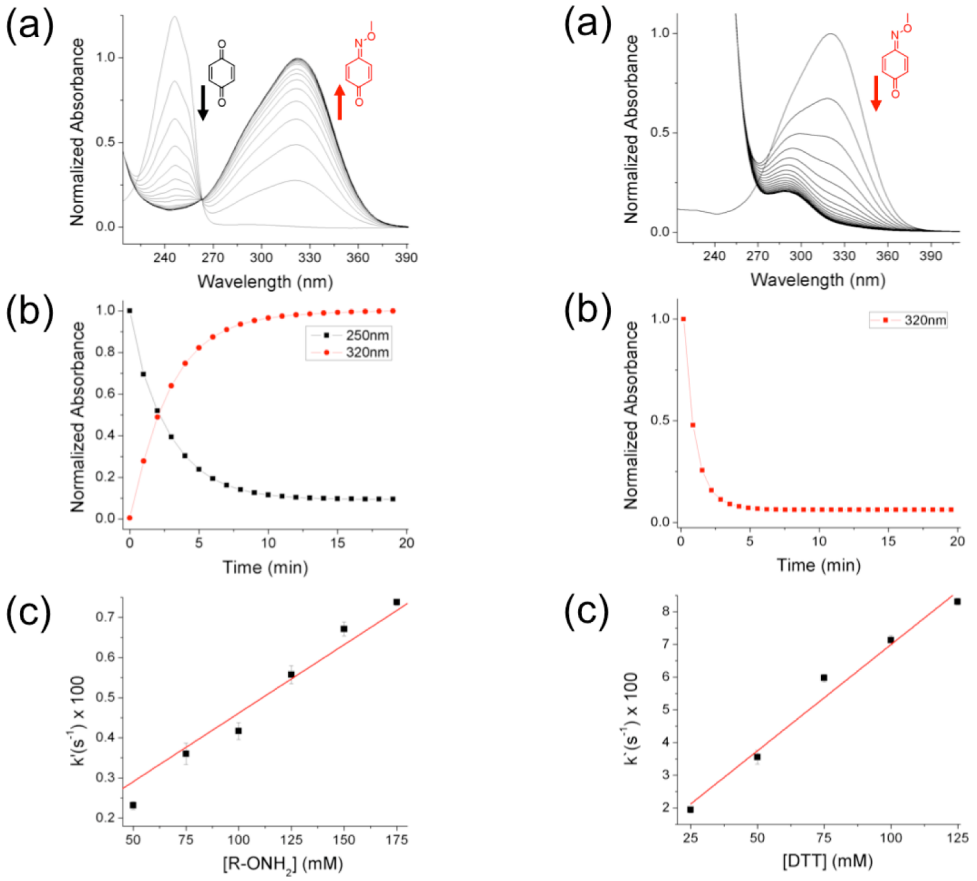

Figure 2. Kinetics of CRRL conjugation between BQ (200 μM) and methoxyamine (100 mM), and cleavage of QO (200 μM) with dithiothreitol (DTT) (100 mM), determined by UV−vis spectroscopy. (Left) (a) UV−vis spectra showing the conversion from BQ (248 nm) to QO (320 nm) with a clear isosbestic point. (b) A plot of absorbance maxima versus time for BQ and QO. An exponential increase of QO and decay of BQ are fitted to pseudo-first-order kinetics to obtain k' (4.2×10^{-3} s^{-1}). (c) A plot of k' versus varying [CH_3−ONH_2] to determine the second-order rate constant k for the reaction of BQ with CH_3ONH_2 to generate QO. The rate constant for CRRL conjugation is 3.4×10^{-2} ($\pm 6.5 \times 10^{-4}$) $M^{-1}s^{-1}$. (Right) (a) UV−vis spectra characterization for the cleavage reaction versus time (QO, 320 nm; AP, 292 nm). (b) A plot of QO (200 μM) absorbance (320 nm) versus time in the presence of the reductant DTT (10 mM) follows a pseudo-first-order exponential decay ($k' = 7.3 \times 10^{-2}$ s^{-1}). (c) A plot of k' versus varying [DTT] to determine the second-order rate constant k for the cleavage of QO to generate AP. The CRRL cleavage rate constant $k = 6.5 \times 10^{-1}$ ($\pm 3.0 \times 10^{-2}$) $M^{-1}s^{-1}$.

2a, right). Although DTT masks the spectrum that is lower than 260 nm, the decrease of QO absorbance at 320 nm and the appearance of AP at 292 nm could be observed. The absorbance peak is shifted from 297 to 292 nm for AP because of the high concentration of DTT. For the case of reduction in low glutathione (GSH) concentration, AP has an absorbance peak at 297 nm (Figure S2, Supporting Information). Commercial AP (200 μM) in 10 mM DTT and PBS also shows the same absorbance shift. The isosbestic point was not observed for the conversion of QO to AP due to a peak absorbance overlap. The rate constant for the CRRL cleavage reaction was determined to be 6.5×10^{-1} (3.0×10^{-2}) $M^{-1}s^{-1}$ (Figure 2c, right). The reaction products from both CRRL conjugation (QO) and cleavage reactions (AP) were characterized by NMR and mass spectrometry (Figures S3 and S4, Supporting Information). These UV characterizations clearly show that the CRRL conjugation and cleavage reactions are well behaved and clean at pH 7.4. We found that the key CRRL conjugation product (QO) is stable at room temperature in PBS for several weeks and could be stored for very long periods (>6 months) at 0 °C (Figure S5, Supporting Information).

To conjugate a range of biomolecules using the CRRL strategy, we used standard solid phase peptide synthesis (SPPS) to generate dynamic peptides containing the hydroquinone group at various locations (Figure 3). We first synthesized a protected hydroquinone-tethered Fmoc amino acid (HQAA; **1**) that could easily be incorporated into a standard solid phase peptide synthesizer for site-specific CRRL chemistry (Figure 3a). A tetrahydro-2*H*-pyran (THP)-protected HQ-tethered alkyl azide (**5**) was synthesized and conjugated to an Fmoc-protected propargyl glycine by 1,3-dipolar cycloaddition. The HQAA (**1**) can be incorporated into any position in a peptide sequence, enabling control of the conjugation site with routine Fmoc SPPS (Figure 3b). The THP group can then be deprotected yielding the HQ using the standard trifluoroacetic acid (TFA) cleavage step for peptide release from a resin with ∼60% yield (0.1 mmol scale; Figure S6, Supporting Information). CRRL's compatibility with facile SPPS is an important feature for generating a range of biomolecules including carbohydrates, polymers, nucleic acids, proteins, and small molecule ligands and nanomaterials that contain the hydroquinone group for a broad range of applications in material science and biology.

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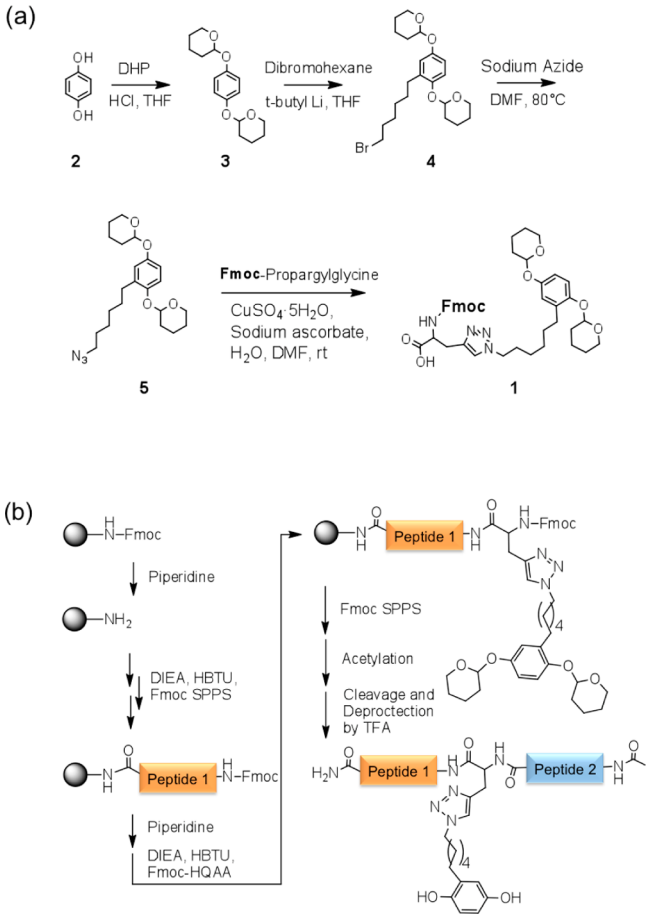

Figure 3. (a) Synthesis of a protected hydroquinone tethered Fmoc amino acid (HQAA; **1**) for use in solid phase peptide synthesis to generate peptides containing the hydroquinone group for site-specific CRRL chemistry. (b) Scheme showing how HQAA (**1**) can be added to any site within a peptide by applying standard solid phase peptide synthesis technology. This strategy allows for the CRRL method to be incorporated into a variety of biomolecules.

In order to demonstrate the utility of the CRRL strategy, we used the well known cell adhesion peptides discovered by Pierschbather and Ruoslahti as a model system. They reported the short peptide sequence Gly-Arg-Gly-Asp-Ser (GRGDS) found in the extracellular matrix protein fibronectin to be a minimum cell adhesion ligand for integrin receptors on cell surfaces.[36] Although GRGDS alone can result in cell adhesion, several other peptide sequences including PHSRN (found in fibronectin within the FIII9 domain) have been shown to have a synergistic or competitive adhesion effect.[37,38] Furthermore, it has been shown that cyclic-RGD has a higher activity toward cell binding than linear-RGD (K_d of nanomolar versus micromolar, respectively) due to the structural similarity between the cyclic form and the natural RGD loop in the FIII10 domain.[39] We chose to focus on the RGD cell adhesive peptide to demonstrate the generation of a dynamic biomolecule using CRRL, which can modulate its biological activity by inducing structural changes upon sensing different redox environments. Two proof-of-concept peptides, intra-CRRL RGD (**6**) and inter-CRRL RGD (**9**), were synthesized to demonstrate the utility of the CRRL methodology (Figure 4).

The intra-CRRL RGD peptide (**6**) has the HQAA (**1**) at the C-terminus and an aminooxy group at the N-terminus (Figure 4a). An added feature of the CRRL strategy is the ease of incorporation of an aminooxy group into a peptide by using a commercially available (Boc-aminooxy)acetic acid, a CRRL ligand, during standard solid phase peptide synthesis. In a reducing environment (1 mM DTT in PBS), the intra-CRRL RGD (**6**) remains in the linear form for more than 4 days (PBS, 25 °C). When molecule **6** (100 μM) encounters oxidative conditions (10 mM of copper(II) sulfate pentahydrate in PBS), the HQ is immediately oxidized to the BQ form, which reacts rapidly with the aminooxy-functionalized N-terminus to generate a cyclized RGD peptide (**7**) (Figure 4b). The cyclized intra-CRRL RGD peptide (**7**) is stable for weeks in PBS and room temperature (25 °C). To regenerate the linearized RGD peptide, the addition of a reductant (10 mM DTT in PBS, 37 °C) to the purified **7** results in instant cleavage of the oxime bond to generate the AP group and a hydroxyl-functionalized N-terminus (**8**) (Figure 4c). As this intra-CRRL peptide has both a CRRL receptor and a ligand, characterization of each state of the peptide was determined by mass spectrometry. This allows for facile monitoring of the chemical changes occurring during the CRRL cyclization and cleavage steps of each species (HQ, oxyamine, QO, AP, and the corresponding hydroxyl terminated group in a single peptide) during the course of the CRRL conjugation and release steps.

Similarly, inter-CRRL formation and cleavage between RGD and PHSRN were characterized by mass spectrometry, as shown in Figure 4 (right). The inter-CRRL RGD peptide (**9**) has a HQAA at the N-terminus for the conjugation of an aminooxy-tethered PREDRVPHSRN peptide. Oxidative conditions (1 mM Cu^{2+}, PBS, 37 °C) immediately yielded the large hybrid peptide (**10**) having a molecular mass of 2479.25, which can then be dissociated by DTT, yielding the corresponding AP-RGD (**11**).

UV−vis spectroscopy also confirmed that the HQ-peptides (**9**) behave similarly to the parent molecules (without peptides attached) (Figure S8, Supporting Information). Intra-CRRL RGD (**6**, Figure S8a, Supporting Information) and inter-CRRL RGD (**9**, Figure S8b, Supporting Information) showed the same HQ absorption spectra associated with each CRRL step. The reduced form of the CRRL peptide (HQ-peptide (**6**, **9**)) was dissolved in H_2O (100 μM) and oxidized by the addition of 2 equivalents of Cu^{2+} to generate QO-peptide (**7**, **10**). For the inter-CRRL peptide, methoxyamine (100 mM, ddH_2O) was added and oxime formation occurred instantly (QO-peptide). The QO-peptide was converted to the AP-peptide (**9**, **11**) by treatment with GSH (5 mM) or DTT (100 mM). The reduced form of the inter-CRRL RGD (HQ-peptide (**9**)) was dissolved in H_2O and oxidized by the addition of 2 equivalents of Cu^{2+} to generate the oxidized BQ-peptide. When methoxyamine (100 mM, ddH_2O) was added, oxime formation occurred instantly (QO-peptide). The QO-peptide was converted to the AP-peptide by treatment with DTT (10 mM). Taken together, these results show that the CRRL strategy can be used to cyclize molecules, conjugate to other molecules, and release molecules responding to environmental redox stimuli with real-time characterization by a noninvasive straightforward UV−vis absorption spectroscopy technique. This facile characterization is important not only for identifying species but for also determining actual amounts of the CRRL species and for monitoring the kinetics and therefore degree of conjugation.

As further chemical characterization, a selected ion chromatogram was performed to demonstrate that each intra-CRRL peptide species behaves similarly to the corresponding

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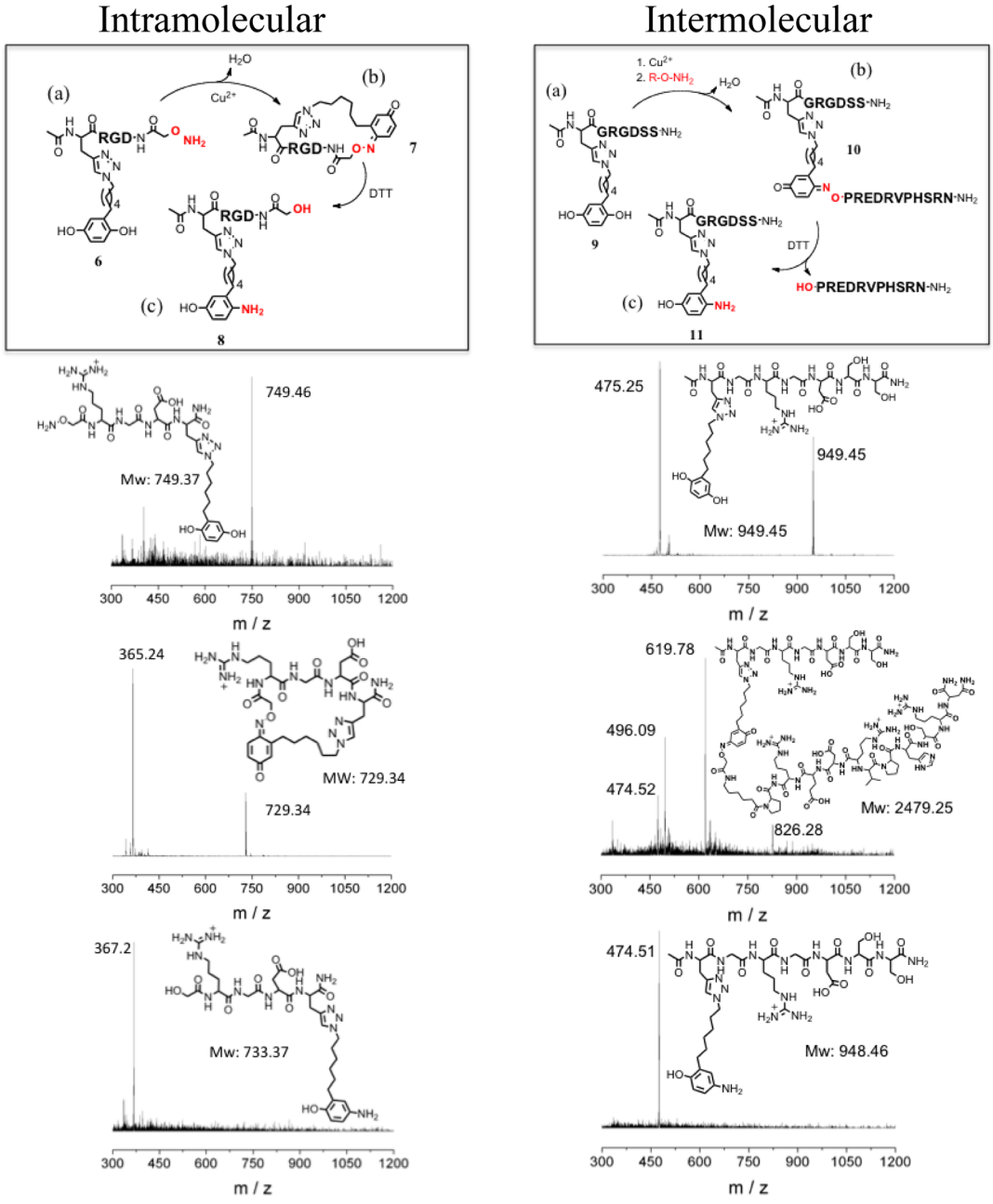

Figure 4. (Left) Intramolecular CRRL. A peptide (intra-CRRL RGD, (**6**)) containing the HQAA (**1**) and a terminal aminooxy group was synthesized using standard solid phase peptide synthesis. The CRRL strategy was used to generate a cyclic peptide characterized by liquid chromatography and mass spectrometry. (a) Structure of the intra-CRRL RGD (**6**) peptide. (b) A mild oxidant Cu^{2+} was added in order to oxidize the HQ to BQ followed by rapid intramolecular oxime formation to generate a cyclic peptide (**7**). (c) A mild reductant DTT was added to the solution of cyclized RGD peptide (**7**) to reduce and therefore cleave the oxime bond to generate the linear peptide (**8**). Mass spectrometry characterization of each product in the intramolecular CRRL strategy are shown. All of the reactions were performed at physiological conditions (PBS, pH 7.4, 37.0 °C). (Right) Intermolecular CRRL demonstrating the conjugation of two different peptides. One peptide (inter-CRRL RGD (**9**)) contains the HQ group, while the other (PREDRVPHSRN) has an oxyamine group. Each are easily synthesized using standard solid phase peptide technology. (a) Inter-CRRL RGD (**9**) peptide in PBS before reaction. (b) A mild oxidant Cu^{2+} and oxyamine-PREDRVPHSRN were added to the PBS solution of inter-CRRL RGD (**9**) peptide to enable rapid intermolecular CRRL conjugation of the two peptides (**10**) (PBS, 37.0 °C). (c) The CRRL product peptide (**10**) was then selectively cleaved upon treatment of the reductant DTT to yield the linear-RGD peptide (**11**). (Bottom) Mass spectrometry characterization of each product in the intermolecular CRRL strategy. All of the reactions were performed at physiological conditions (PBS, pH 7.4, 37.0 °C).

parent molecule without the peptide (Figure 4), in terms of the elution durations from a C18 column (Figure S7, Supporting Information).

As a demonstration to show the utility of the inter- and intra-CRRL-generated peptides (**6**–**11**), we performed a standard competitive inhibition assay for cell adhesion.[37,38] This assay involves the pretreatment of cells suspended in solution with soluble inhibitors and then seeding the cells to a tailored and biospecific ligand surface to evaluate the ability of the soluble inhibitors on cell adhesion (Figure 5).

dx.doi.org/10.1021/bc400565y | *Bioconjugate Chem.* 2014, 25, 543−551

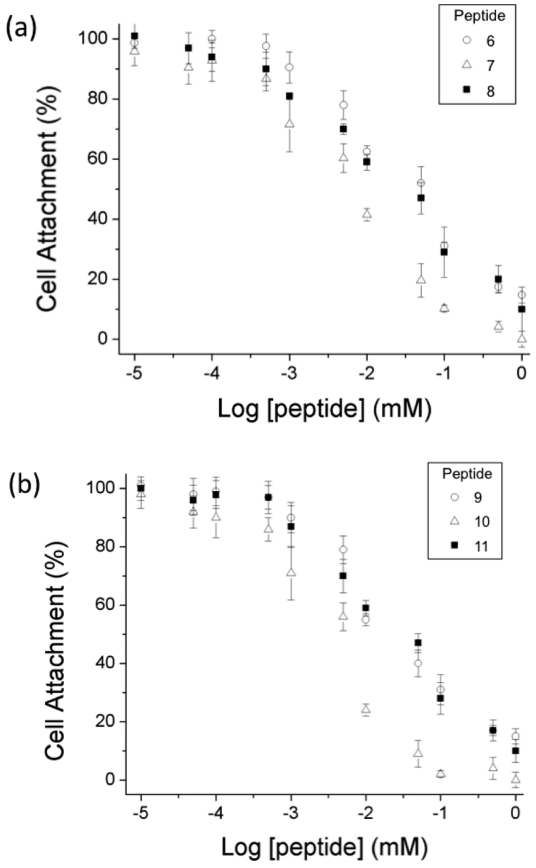

Figure 5. Cell adhesion inhibition assay for inter- and intra-CRRL peptides (**6**–**11**). Swiss Albino 3T3 fibroblasts were suspended in serum-free media and incubated with peptides **6**–**8** (a) and **9**–**11** (b) at various concentrations (10 nM to 1 mM for 5 min at 37 °C). The pretreated cells were then seeded and incubated onto a biospecific cell adhesive RGD presenting self-assembled monolayer surface.[22] The cells that adhered were counted, and the ratios (%) of adhered cells to the nonpretreated cell control were tabulated. (a) Cyclic form of intra-CRRL RGD (cyclic RGD (**7**) open triangle) shows higher cell inhibition than the two linear forms of RGD for the intra-CRRL RGD (**6** and **8**, circle and square). (b) PHSRN conjugated inter-CRRL RGD (dual ligand hybrid (**10**) open triangle) shows higher cell inhibition activity than the single ligand nonconjugated forms (**9** and **11**, circle and square).

Each of the peptides was incubated (pretreated) with Swiss 3T3 fibroblasts in solution at varying concentrations, and then the cells were seeded to self-assembled monolayer (SAM) surfaces presenting an immobilized linear-RGD peptide.[23] It has been shown previously that these surfaces are ideal for evaluating small molecules that perturb cell adhesion.[25] Without pretreatment with the peptides (**6**–**11**), the cells efficiently attached to the RGD surfaces through integrin receptors via biospecific interactions.

Figure 5 shows the cell adhesion inhibition assay as a percent ratio of each peptide with the nonpretreated cells. As expected, the cyclic intra-CRRL peptide (cyclic RGD, **7**) and the inter-CRRL hybrid peptide that contains RGD and PHSRN ligands (**10**) were the best inhibitors for cell adhesion due to their higher affinity for integrin receptors than molecules **8**, **9**, and **11**, which are linear forms of the lower affinity RGD peptide for integrin receptors.[37,38] Additionally, QO offers an alternative to disulfide bonds as a cleavable linker between two target biomolecules. We determined that CRRL cleavage takes 10 h for reaction completion in the presence of 5 mM glutathione (GSH) in PBS (Figure S2, Supporting Information).

As a second demonstration, we utilized the CRRL methodology to deliver cargoes to cells via an intracellular redox cleavage (Figure 6). We took advantage of the reducing

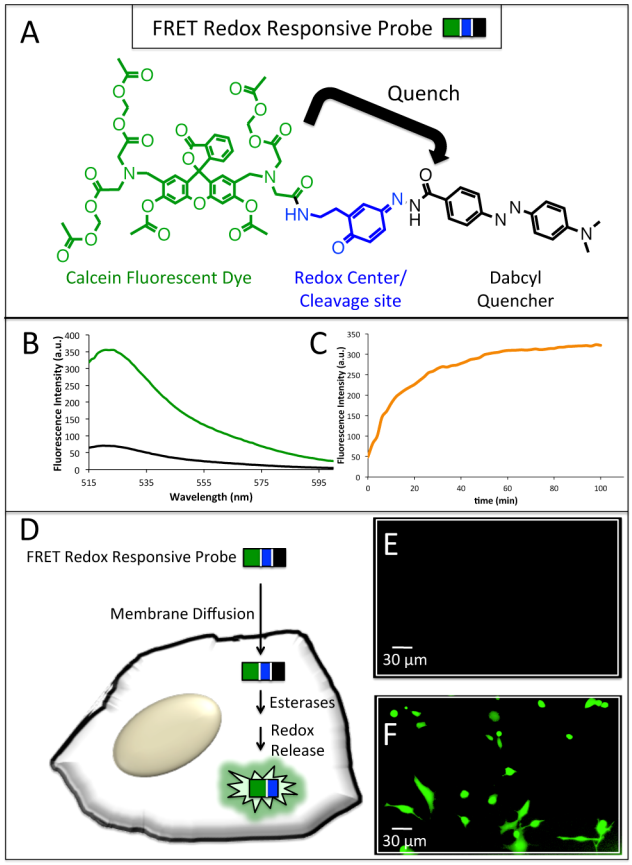

Figure 6. CRRL strategy to release cargo in cells via an intracellular redox trigger. (A) Structure of a cell-permeable redox-responsive calcein dye (CPRRC). The molecule has three distinct parts. The calcein dye is capped to allow the highly charged dye to cross the cell membrane. The quinone redox center is stable but is cleaved upon encountering the reducing conditions within a cell. The dabcyl group quenches the calcein fluorescence via FRET until it is released by the redox center in the cell cytoplasm. (B) A fluorescence intensity vs wavelength plot for CPRRC before (black line) and after (green line) exposure to 5 mM glutathione solution for 2 h. (C) A fluorescence intensity vs time plot for CPRRC dissolved in 5 mM glutathione solution. Upon release of the dabcyl quencher, the fluorescence intensity increases. (D) Cartoon describing the delivery and cleavage of CPRRC. (E) Fluorescent image of cells before CPRRC exposure (no fluorescence). (F) Image of cells after 1 h of 10 μM CPRRC exposure shows bright fluorescence indicating that CPRRC is cleaved within cells in a redox-responsive manner.

environment found within a cell's cytoplasm to perform a selective *in vivo* CRRL cleavage. In order to show this *in vivo* application, we first synthesized a FRET redox-responsive probe molecule (Figure 6A). This cell-permeable redox-responsive molecule has three components: (1) a calcein dye that is fluorescent; (2) a redox center/cleavage site based upon the quinone group; and (3) a dabcyl group that efficiently quenches fluorescence. The cell-permeable redox-responsive calcein dye (CPRRC) molecule is initially not fluorescent but becomes fluorescent upon reductive cleavage and release of the

dx.doi.org/10.1021/bc400565y | *Bioconjugate Chem.* 2014, 25, 543–551

dabcyl quenching group. It is important to note that the CRRL methodology works for either oxime or hydrazone linkages to quinone groups.

Many commercially available ligands have oxyamine or hydrazine tethers, and this provides tremendous synthetic flexibility for the CRRL methodology when conjugating and cleaving a variety of ligands in a redox manner. Figure 6B shows the difference in fluorescence for the CPRRC molecule before and after (the dabcyl quenching group is redox released) exposure to cell cytoplasm-reducing conditions (5 mM of glutathione) for 2 h. Figure 6C shows the CPRRC fluorescence intensity profile versus time in the presence of 5 mM glutathione and indicates that the quinone redox center is cleaved causing the release of the FRET quenching dabcyl group, which in turn leads to an increase in fluorescence due to the unquenched calcein dye.

To test whether the CRRL methodology can selectively work inside cells, we added the CPRRC molecule to cells in culture (Figure 6D). Untreated cells showed no fluorescence, while treated cells (10 μM CPRRC in cell culture media, 1 h) showed bright fluorescence. As a control, a calcein−dabcyl conjugate that does not contain the redox center showed no fluorescence in the same conditions for the cell culture experiments (data not shown). Taken together, these results show that the CPRRC molecule is efficiently cleaved in the cell cytoplasm reductive environment. By incorporating a range of molecules that are tethered through the quinone redox center, many biomolecules, imaging agents, and nanoparticles may be selectively released within a cell. Furthermore since the CRRL methodology is bio-orthogonal, ligations and cleavage reactions may be performed within cells or complex protein mixtures. Finally, the *in vivo* CRRL strategy may also act as a redox probe to evaluate the cell's response to changing stimuli, which may elicit changes in the local redox environment.

CONCLUSIONS

In summary, we introduce a chemoselective redox-responsive ligation and release strategy. The CRRL strategy is a new class of conjugation chemistry in that it allows chemoselective conjugation and cleavage that is responsive to specific redox stimuli. CRRL is based on the ability of a hydroquinone to oxidize to a benzoquinone for subsequent reaction with oxyamine-tethered ligands to generate a stable oxime product. The oxime can then undergo irreversible cleavage in a reductive environment to generate an aminophenol and a primary hydroxyl-terminated ligand. The aminophenol is stable and can be brought back to the original hydroquinone with mild redox chemistry for subsequent rounds of conjugation and release. The CRRL strategy can be monitored in real-time by standard UV−vis absorption spectroscopy and modulated by chemical or electrochemical methods. A non-natural amino acid containing the hydroquinone group was synthesized and shown to incorporate at selective positions within a peptide by using standard solid phase peptide synthesis. As a demonstration, intermolecular and intramolecular CRRL strategies were used to generate dynamic cyclic peptides and two tethered peptides that could be conjugated and cleaved. This hydroquinone amino acid can easily be incorporated to generate redox-responsive peptide and polymers. As a second demonstration, a FRET redox-responsive probe was synthesized and shown to undergo selective redox cleavage upon intracellular uptake. This strategy may provide new ways to deliver and monitor biomolecules and cellular events based on the redox environment. The CRRL methodology not only shares all of the advantages of traditional oxime chemistry (bio-orthogonal, high-yield, and chemoselective) but also has a dynamic attribute associated with conjugation and cleavability. CRRL conjugation and cleavage occurs quantitatively and in physiological conditions without the need for a traditional catalyst. Furthermore, the CRRL strategy is synthetically flexible and can also be used with hydrazine type molecules instead of aminooxy groups to generate conjugated hydrazones, which also undergo the CRRL cycle.[40] CRRL is a general methodology for conjugation and release and will be a useful tool for scientists and engineers in various fields aiming to design smart molecules and materials, which alter their biological activities according to specific changes in their physiological environments, or for material science applications.[41] The CRRL strategy is inexpensive, dynamic, kinetically well behaved, synthetically modular, and straightforward to monitor and control. To our knowledge, the CRRL strategy is the only single method that is able to chemoselectively ligate molecules and to then release the molecules under redox control with regeneration for potential multiple subsequent rounds of conjugation and release. Future research will explore how the CRRL strategy may be used to release or conjugate molecules to generate dynamic/smart polymers for material science, nanoscience, and tissue engineering applications. For more biological applications, dynamic carbohydrates and mixed CRRL biomolecules (peptides, nucleic acid, and lipids) may be synthesized and selectively released *in vitro and in vivo* for a range of diagnostic/imaging or direct delivery of therapeutics.[42−44] We believe, the ease of the CRRL strategy should find wide use in a range of applications in biology, tissue engineering, nanoscience, and material science and will expand the suite of current conjugation and release strategies.

ASSOCIATED CONTENT

Supporting Information

Experimental details, synthetic schemes, and procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Brian Matthew, Jim Jorgenson, Hal Kohn, David Lawrence (UNC Chapel Hill), and Phil Baran (Scripps) for helpful discussions. This work was supported by the Carolina Center for Cancer Nanotechnology Excellence (NCI), The Burroughs Wellcome Foundation (Interface Career Award), The National Science Foundation (Career Award), National Science and Engineering Research Council of Canada (NSERC), and The Canadian Foundation for Innovation (CFI).

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