J|A|C|S ARTICLES

Published on Web 12/03/2010

Carbon Monoxide-Releasing Micelles for Immunotherapy

Urara Hasegawa, André J. van der Vlies, Eleonora Simeoni, Christine Wandrey, and Jeffrey A. Hubbell*

Institute of Bioengineering (IBI) and Institute of Chemical Sciences and Engineering (ISIC), Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne CH-1015, Switzerland

Received August 19, 2010; E-mail: jeffrey.hubbell@epfl.ch

Abstract: With the discovery of important biological roles of carbon monoxide (CO), the use of this gas as a therapeutic agent has attracted attention. However, the medical application of this gas has been hampered by the complexity of the administration method. To overcome this problem, several transition-metal carbonyl complexes, such as $Ru(CO)_3Cl(glycinate)$, $[Ru(CO)_3Cl_2]_2$, and $Fe(\eta^4\text{-2-pyrone})(CO)_3$, have been used as CO-releasing molecules both in vitro and in vivo. We sought to develop micellar forms of metal carbonyl complexes that would display slowed diffusion in tissues and thus better ability to target distal tissue drainage sites. Specifically, we aimed to develop a new CO-delivery system using a polymeric micelle having a $Ru(CO)_3Cl(amino\ acidate)$ structure as a CO-releasing segment. The CO-releasing micelles were prepared from triblock copolymers composed of a hydrophilic poly(ethylene glycol) block, a poly(ornithine acrylamide) block bearing $Ru(CO)_3Cl(ornithinate)$ moieties, and a hydrophobic poly(*n*-butylacrylamide) block. The polymers formed spherical micelles in the range of 30−40 nm in hydrodynamic diameter. Further characterization revealed the high CO-loading capacity of the micelles. CO-release studies showed that the micelles were stable in physiological buffer and serum and released CO in response to thiol-containing compounds such as cysteine. The CO release of the micelles was slower than that of $Ru(CO)_3Cl(glycinate)$. In addition, the CO-releasing micelles efficiently attenuated the lipopolysaccharide-induced NF-κB activation of human monocytes, while $Ru(CO)_3Cl(glycinate)$ did not show any beneficial effects. Moreover, cell viability assays revealed that the micelles significantly reduced the cytotoxicity of the $Ru(CO)_3Cl(amino\ acidate)$ moiety. This novel CO-delivery system based on CO-releasing micelles may be useful for therapeutic applications of CO.

Introduction

Carbon monoxide (CO), one of the byproducts of heme catabolism via heme oxygenase-1 (HO-1), has recently been recognized as an important signaling mediator in mammals[1,2] despite its bad reputation as an air pollutant. CO plays versatile roles in tissue protection via anti-inflammatory, antiproliferative, and antiapoptotic effects.[1,2] Anti-inflammatory effects have attracted particular growing attention due to possible applications to many inflammation-based diseases, and an increasing number of reports concerning the mechanism through which CO exerts anti-inflammatory effects have appeared.[3−9] In lipopolysaccharide (LPS)-challenged mouse macrophages and human dendritic cells, CO has been shown to inhibit the production of pro-inflammatory cytokines and increase production of anti-inflammatory cytokines.[4−7] In addition, CO suppresses T cell proliferation.[8] Though the mechanism is still not fully understood, it seems that CO elicits these anti-inflammatory effects through p38 MAPK and/or JNK pathways.[4,6] Moreover, the effect of CO has also been confirmed in various disease models such as sepsis, chronic graft rejection, and ischemia/reperfusion injury.[3,4,9] Thus, CO appears to have a high potential utility for future therapeutic applications.

Despite the interesting biological activities, the development of methods for delivering CO is in its infancy. Since CO is toxic at high concentrations, the precise control of the location, timing, and dosages of CO is the critical factor for sufficient therapeutic responses. In addition, the fact that CO is a gaseous molecule makes its controlled delivery more challenging. The simplest administration method is the inhalation of CO at a known concentration. However, this method is a very nonspecific approach that can cause adverse effects. These difficulties led chemists to explore molecules capable of releasing CO in biological systems (CO-releasing molecules) for safer and more

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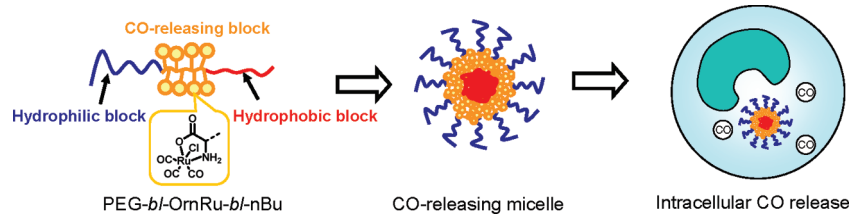

Figure 1. Schematic illustration of micelle formation of PEG-*b*-OrnRu-*b*-nBu triblock copolymer and CO release after cellular uptake.

convenient gas-delivery systems. The pioneering work of Motterlini and co-workers showed that transition-metal carbonyl complexes such as $Ru(CO)_3Cl$(glycinate), $[Ru(CO)_3Cl_2]_2$, and Fe(η^4-2-pyrone)$(CO)_3$ had high potential as CO-releasing molecules.[10−14] Among them, $Ru(CO)_3Cl$(glycinate) and its derivatives ($Ru(CO)_3Cl$(amino acidate)) are very promising water-soluble CO-releasing molecules.[15] In particular, the pharmacological actions of $Ru(CO)_3Cl$(glycinate) have been extensively studied. This compound has been shown to alleviate damage in ischemia/reperfusion injury, inhibit allograft rejection, suppress nitric oxide (NO) production from macrophages, and attenuate cardiovascular inflammation and thrombin-induced neuroinflammation.[11,16−18]

Though the discovery of these CO-releasing molecules opens up new possibilities, there are still several issues to overcome for medical applications, particularly those in which downstream tissue sites draining the injection site are targeted. These small molecular drugs diffuse rapidly within the body after administration and may liberate CO prior to reaching these target tissues. Thus, there is a considerable need for developing a safe and efficient CO-delivery system.

In the field of drug delivery, polymeric nanocarriers are recognized as a powerful system for delivering a wide variety of therapeutic agents such as hydrophobic drugs, nucleotides, and proteins.[19−22] It has been shown that the precise size control in the subhundred nanometer range enables targeted delivery to specific tissues such as tumor tissues and lymph nodes.[23−25] In particular, the use of polymeric micelles, spherical supramolecular assemblies from amphiphilic block copolymers, as drug carriers is a very promising method due to their unique characteristics such as high drug-loading capacity, easy formulation, and low toxicity. Taking these advantages into consideration, we aimed to develop a new gas-delivery system based on a polymeric micelle.

Recently, our laboratory reported a micelle-based gas-delivery system for NO, the first gaseous species to be identified as a cell signaling agent.[26] The NO-releasing micelles have a core composed of *N*-diazenium diolate (NONOate) moieties, which decompose to liberate NO in the presence of protons. The micelles showed remarkably prolonged NO release due to the slow diffusion of protons into the NONOate core. This promising result prompted us to develop a second gas-delivery micelle system for CO.

We report here a novel CO-delivery system using a polymeric micelle as a CO carrier. The CO-releasing micelles were prepared from triblock copolymers composed of a hydrophilic poly(ethylene glycol) block, a poly($Ru(CO)_3Cl$(ornithinate acrylamide) block capable of releasing CO, and a hydrophobic poly(*n*-butylacrylamide) block. The micelles were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), and analytical ultracentrifugation (AUC). The CO-release property was examined, and possible biological molecules capable of inducing CO-release were identified. In addition, the anti-inflammatory effect and cytotoxicity of the CO-releasing micelles were assessed with a human monocyte-derived cell line.

Results and Discussion

Synthesis of PEG-*b*-OrnRu-*b*-nBu Triblock Copolymer. Several transition-metal carbonyl complexes have been reported as sources of CO and have been shown to attenuate inflammation, promote wound healing, and reduce transplant rejection. Among them, $Ru(CO)_3Cl$(glycinate), which is synthesized by ligand substitution between $[Ru(CO)_3Cl_2]_2$ and glycine, is one of the most promising CO-releasing molecules. To incorporate this $Ru(CO)_3Cl$(amino acidate) structure into a micelle, we designed a triblock copolymer of poly(ethylene glycol)-*b*-poly[$Ru(CO)_3Cl$(ornithinate acrylamide)]-*b*-poly(*n*-butylacrylamide) (PEG-*b*-OrnRu-*b*-nBu), where PEG is the hydrophilic block used to stabilize the micelle, OrnRu is a $Ru(CO)_3Cl$(ornithinate) block that releases CO, and nBu is the hydrophobic block, which drives micellization (Figure 1).

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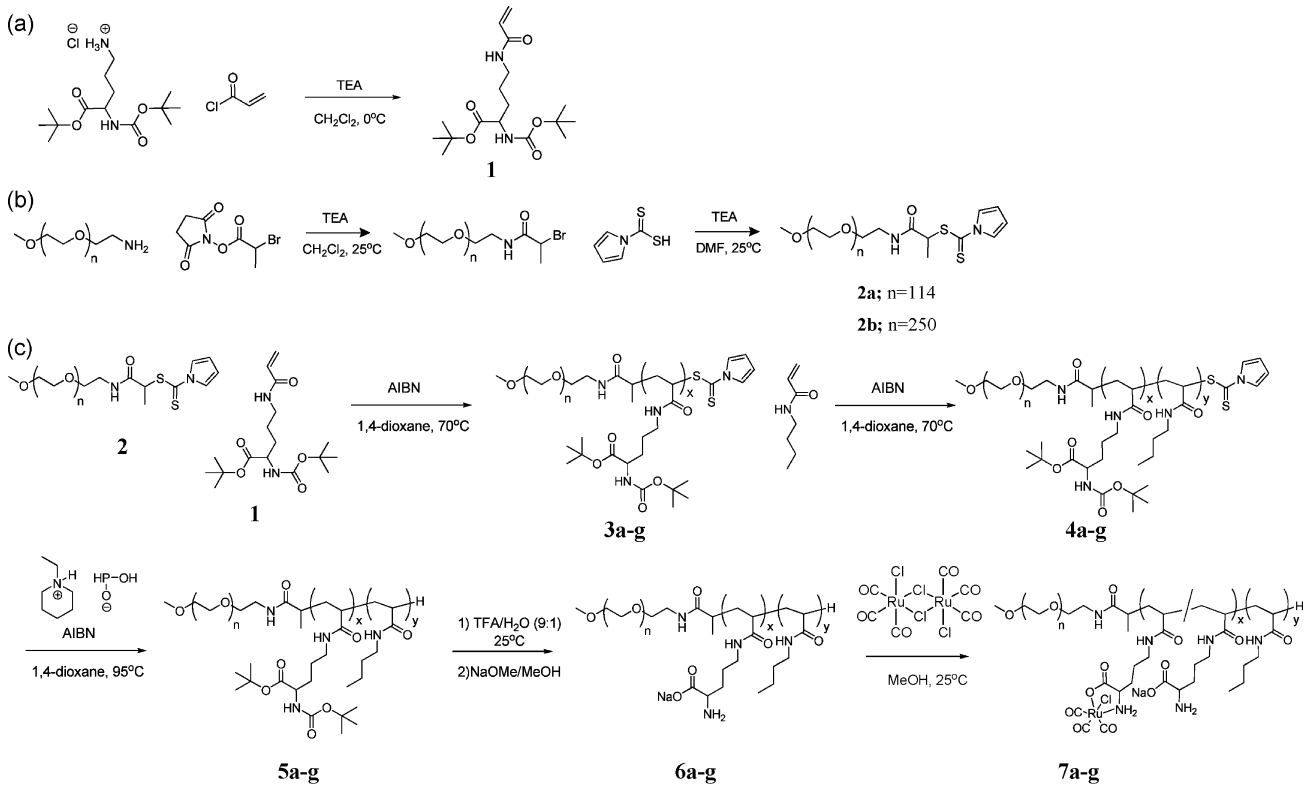
Scheme 1. Synthesis of (a) Boc-Orn-OtBu AAm, (b) PEG-CTA, and (c) PEG-*b*-OrnRu-*b*-nBu Triblock Copolymers



The PEG-*b*-OrnRu-*b*-nBu triblock copolymers were prepared as shown in Scheme 1. First, PEG-*b*-(Boc-Orn-OtBu)-*b*-nBu-CTA triblock copolymers **4a**−**g** were synthesized by reversible addition−fragmentation transfer (RAFT) polymerization. The degree of polymerization of each segment was successfully controlled by changing the monomer/chain-transfer agent (CTA) ratio (Tables S1 and S2, Supporting Information). Gel permeation chromatography (GPC) showed unimodal size distributions of PEG-*b*-(Boc-Orn-OtBu)-CTA diblock copolymers **3a**−**g** (Figures S6 and S7a, Supporting Information). In the case of PEG-*b*-(Boc-Orn-OtBu)-*b*-nBu-CTA triblock copolymers **4a**−**g**, a prominent peak with a small shoulder in the higher molecular weight region was observed (Figure S7b). Thereafter, the CTA end group was removed by radical-induced reduction with 1-ethylpiperidine hypophosphite (EPHP).[27] Successful removal of the end group was confirmed by the absence of the peaks corresponding to the pyrrole protons in the ^{1}H NMR spectrum (Figure S4b, Supporting Information). After CTA removal, the shoulder peak at higher molecular weight was no longer detectable in the GPC trace (Figure S7c). The facts that (1) the high molecular weight peak in the GPC trace has an absorbance around 300 nm, which is similar to the $\pi-\pi^*$ transition of the dithioester group and (2) a monomodal molecular weight distribution was measured by GPC after the radical-induced reduction suggest that the high molecular weight peak consists of two polymers linked by a dithioester-containing group and the radical-induced reduction reaction apparently is capable of cleaving this linkage as shown by the disappearance in the GPC trace. (For further discussion, see the Supporting Information).

Next, the Boc and OtBu protecting groups were removed in trifluoroacetic acid (TFA) and treated with sodium methoxide (NaOMe) to convert the Orn segment to the sodium salt form followed by Sephadex LH-20 column chromatography purification. The absence of the peaks corresponding to the protecting groups was confirmed by ^{1}H NMR (Figure S5b, Supporting Information).

Finally, the obtained PEG-*b*-OrnNa-*b*-nBu triblock copolymers **6a**−**g** were complexed with $[Ru(CO)_3Cl_2]_2$ and purified by Sephadex LH-20 column chromatography. IR spectra of the polymers showed the three characteristic bands in the carbonyl vibration region similar to those of $Ru(CO)_3Cl$(glycinate) (Table 1 and Figure S8, Supporting Information). The broadening of the signals corresponding to the Orn block in ^{1}H NMR spectra indicates the complexation between the OrnNa block and $[Ru(CO)_3Cl_2]_2$ (Figure S5b, Supporting Information). To obtain the degree of conversion of OrnNa to OrnRu, the ruthenium content of PEG-*b*-OrnRu-*b*-nBu polymers **7a**−**g** was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). As shown in Table 1, the conversion of the OrnNa unit to an OrnRu unit was 58−75% possibly due to the bulkiness of the $Ru(CO)_3Cl$(ornithinate) group that interferes with the full complexation.

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Table 1. Characterization of PEG-*b*-OrnRu-*b*-nBu Triblock Copolymers

sample	ν(CO)[a] (cm^{-1})	Ru concn[b] (wt %)	no. of OrnRu units per polymer	degree of Ru complexation[c] (%)
7a	2135, 2049, 1968	9.2	20	75
7b	2134, 2047, 1968	10.0	24	65
7c	2134, 2047, 1968	12.9	37	70
7d	2134, 2050, 1968	6.9	18	70
7e	2134, 2049, 1970	4.7	15	58
7f	2134, 2048, 1970	8.9	27	63
7g	2134, 1046, 1968	10.3	37	72
$Ru(CO)_3Cl$(glycinate)	2135, 2045, 1981	34.6		102

[a] Determined by ATR-IR. [b] Determined by ICP-OES. [c] Degree of Ru complexation = [OrnRu]/([OrnNa] + [OrnRu]).

Table 2. Characterization of PEG-*b*-OrnRu-*b*-nBu Micelles

	hydrodynamic diameter[a]					
sample	*z* average (nm)	PDI	partial specific volume[b] (mL/g)	molar mass of the micelle[c] (g/mol)	aggregation number (no. of chains/micelle)	no. of OrnRu units per micelle
7a	29	0.19				
7b	33	0.21				
7c	32	0.19	0.7893	1.82×10^6	63	2.3×10^3
7d	37	0.15	0.8536	1.79×10^6	66	1.2×10^3
7e	41	0.11				
7f	44	0.14				
7g	39	0.12	0.8568	3.14×10^6	88	3.3×10^3

[a] *z* average and polydispersity index (PDI = μ_2/Γ^2) calculated by the cumulant method (DLS). [b] Determined by densitometry. [c] Weight average of *c*(M) calculated by SEDFIT.

Micelle Formation from PEG-*b*-OrnRu-*b*-nBu Triblock Polymers. To prepare CO-releasing micelles, we first designed PEG-*b*-OrnRu diblock copolymers assuming that the OrnRu block would be hydrophobic enough to drive micelle formation. However, in contrast to our expectation, the diblock copolymers formed polydisperse aggregates larger than 200 nm (data not shown). This might be due to the bulkiness of the ruthenium carbonyl group, which may disfavor formation of a compact structure. Moreover, its complicated solution chemistry[28] as well as the remaining OrnNa residues may make the OrnRu segment less hydrophobic than expected. This initial observation, therefore, led us to design a new block copolymer having a hydrophobic third segment, an nBu block, for driving micelle formation.

DLS showed that the triblock copolymers formed monodisperse micelles about 30 nm in diameter for the polymers with 5 kDa PEG (**7a**, **7b**, **7c**) and about 40 nm in diameter for the polymers with 11 kDa PEG (**7d**, **7e**, **7f**, and **7g**) (Table 2). The micelles had a high colloidal stability in distilled water, and the aggregation was not observed for 1 week at both room temperature and 4 °C (Figure S9, Supporting Information). TEM images showed that all triblock copolymers formed spherical micelles (Figure 2). Generally, block polymers with a large hydrophilic segment form spherical micelles, whereas those with a smaller hydrophilic segment tend to form wormlike micelles or vesicles.[29] We expected to observe morphological changes for the polymers composed of short hydrophilic blocks (PEG) and long hydrophobic blocks (OrnRu and nBu), such as **7c**. However, the transition from spherical micelles to other structures was not observed for the PEG-*b*-OrnRu-*b*-nBu triblock polymers under the ratios of block sizes explored.

The micelles (**7c**, **7d**, and **7g**) were further characterized by sedimentation velocity measurements. A typical sedimentation coefficient distribution, *c*(s), and a molar mass distribution, *c*(M), are shown in Figure 3 for the **7d** micelle. Figure 3a shows a prominent unimodal peak with a maximum near 20 S (90% of the total absorbance) (S = Svedberg = 10^{-13} s), a small amount with lower sedimentation coefficients ($s_{w,20}$) (about 6% of the total absorbance), and about 4% at higher $s_{w,20}$ values. Concentration independence of *c*(s) was confirmed by sedimentation velocity for all three micelles studied (Table S3, Supporting Information). After conversion of *c*(s) into *c*(M), the aggregation number and the number of OrnRu units of the micelles were estimated from the average molar mass value. As shown in Table 2, the number of OrnRu units of the **7c**, **7d**, and **7g** micelles were 2300, 1200, and 3300 per micelle, respectively. Assuming that each OrnRu unit releases 1 equiv of CO, the **7c**, **7d**, and **7g** micelles can deliver 2300, 1200, and 3300 equiv of CO per micelle.

The spherical nature of the micelles was confirmed by UltraScan two-dimensional spectrum analysis (2DSA) and subsequent Monte Carlo analysis (2DSA−MC), yielding a frictional ratio (f/f_0) of about 1.1 for the main fraction. The plots of the frictional ratios (f/f_0) versus $s_{w,20}$ are presented for three concentrations of the **7d** micelles (Figure S10, Supporting Information). Moreover, the sedimentation coefficient range of the main fraction of the **7d** micelles is almost identical with the range obtained by SEDFIT and shown in Figure 3.

CO-Release Property of the Micelles. It is believed that $Ru(CO)_3Cl$(glycinate) releases CO in the body via substitution of chloride or glycinate ligands with electron-withdrawing ligands such as thiol compounds and imidazole.[30] Although the precise mechanism is not known, the successful results observed in vitro and in vivo also imply that $Ru(CO)_3Cl$(glycinate) releases a sufficient amount of CO in the physiological milieu.[31] To quantify the amount of CO release in aqueous medium, the spectrophotometric change of deoxymyoglobin (Mb) to carboxy-Mb (MbCO) has often been used.[10] In this assay, the sample solution is added to the Mb solution containing sodium dithionite ($Na_2S_2O_4$) as a reducing agent, and then the formation of MbCO is quantified. It has been confirmed that $Ru(CO)_3Cl$(glycinate) liberates 1 equiv of CO with a half-life of ~1 min.[11] Thus, we first used the Mb assay to examine the CO-releasing capacity of the **7g** micelles. The micelles released 0.76 equiv of CO per OrnRu unit, whereas $Ru(CO)_3Cl$(glycinate) released 0.90 equiv of CO (Figure S11b, Supporting Information). This slightly lower CO release from the micelles can be attributed to the micelle structure, which keeps some $Ru(CO)_3Cl$(ornithinate) moieties intact inside the micelles. Considering that the **7g** micelles have 3300 OrnRu units according to the AUC analysis, the CO-loading capacity of the micelles is calculated to be 2500 equiv of CO per micelle. This high loading will be advantageous to enhance the therapeutic efficiency of CO.

Though we confirmed that the micelles are able to release CO using the Mb assay, the CO-release mechanism under the physiological conditions is still not clear. In particular, further investigation is needed to indentify molecules capable of

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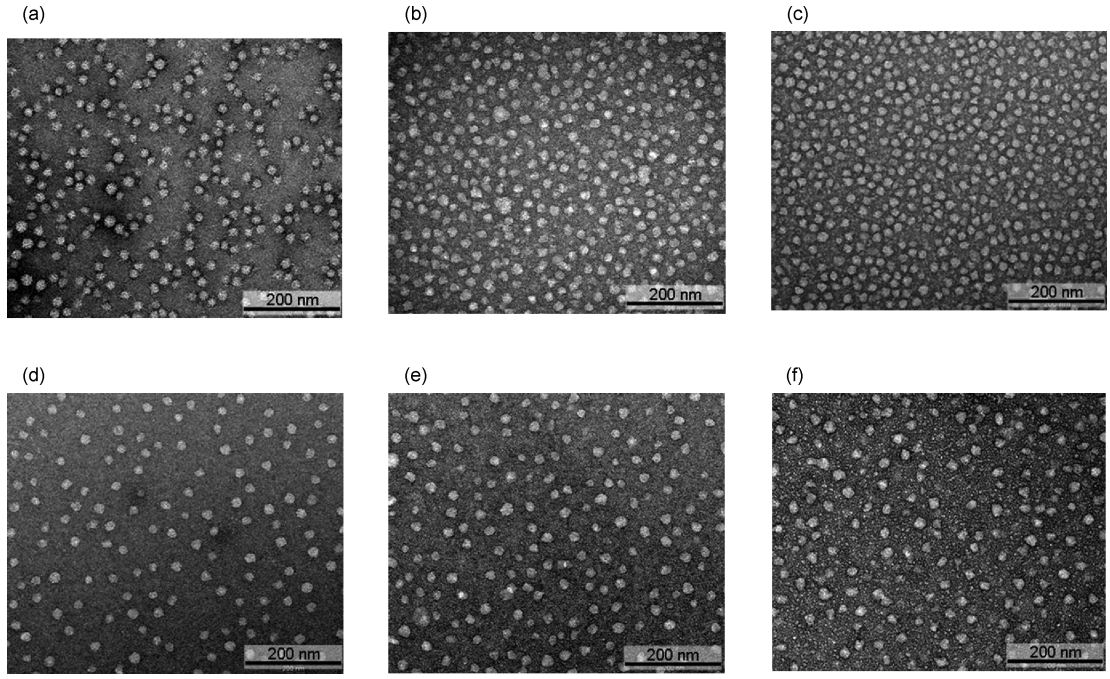

Figure 2. TEM images of PEG-*b*-OrnRu-*b*-nBu micelle formulations: (a) **7a**, (b) **7b**, (c) **7c**, (d) **7d** (e) **7e**, (e) **7g**. Samples were negatively stained with 2 wt % uranyl acetate(aq).

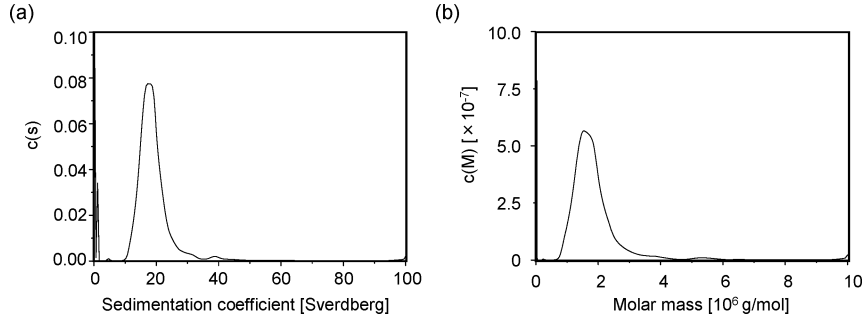

Figure 3. Typical (a) sedimentation coefficient distribution and (b) molar mass distribution of PEG-*b*-OrnRu-*b*-nBu **7d** micelles (sedimentation velocity at 30 000 rpm, 20 °C, $\lambda = 270$ nm, micelle concentration 1 mg/mL).

inducing CO release in the body. Since $Na_2S_2O_4$ does not exist in the body and Mb mainly localizes in muscle tissues,[32] it is not appropriate to consider the data obtained by this method as a real CO-release profile in the body. Furthermore, $Na_2S_2O_4$ induces CO liberation of $Ru(CO)_3Cl$(glycinate) (see Figure S13c in the Supporting Information). Therefore, we sought to find a method to measure CO release without any additives in physiologically relevant conditions.

The experiments were carried out in a desiccator equipped with a CO detector and a test tube for loading a sample solution (Figure S12, Supporting Information). Assuming that the gas and liquid phases reach equilibrium and the pressure in the desiccator stays at 1 atm, one can calculate the amount of released CO (N_{CO}) using the following equation:

$$N_{CO} = \frac{pV_g}{RT} + cV_l = p\left(\frac{V_g}{RT} + \frac{V_l}{k}\right) \quad (1)$$

where p is the partial pressure of CO, V_g and V_l are the volume of the gas phase (250 mL) and liquid phase (5 mL), R is the gas constant, T is the temperature, c is the CO concentration in the liquid phase, and k is Henry's law constant of CO in water (1052.63 (L·atm)/mol at 25 °C).

Since thiol compounds such as cysteine, glutathione, and proteins with free cysteine residues are abundant inside cells,[33] these molecules may be potential candidates to induce CO release in the body. Therefore, we focused on the effects of cysteine on CO release from $Ru(CO)_3Cl$(glycinate) and the CO-releasing micelles. As shown in Figure 4a, CO release by $Ru(CO)_3Cl$(glycinate) was dependent on the cysteine concentrations. In the presence of 10 mM cysteine, the amount of released CO increased to 0.12 equiv of CO per molecule within 1.5 h, whereas almost no CO release was observed in the presence of 0.1 mM cysteine for 2 h. Moreover, after incubation of $Ru(CO)_3Cl$(glycinate) with 1 mM cysteine, the addition of 10 mM cysteine induced additional CO release (Figure S13a, Supporting Information). These data clearly show that cysteine is able to interact with $Ru(CO)_3Cl$(glycinate) and subsequently induces CO release. The micelles showed a similar CO-release profile but with slower release rates than that of

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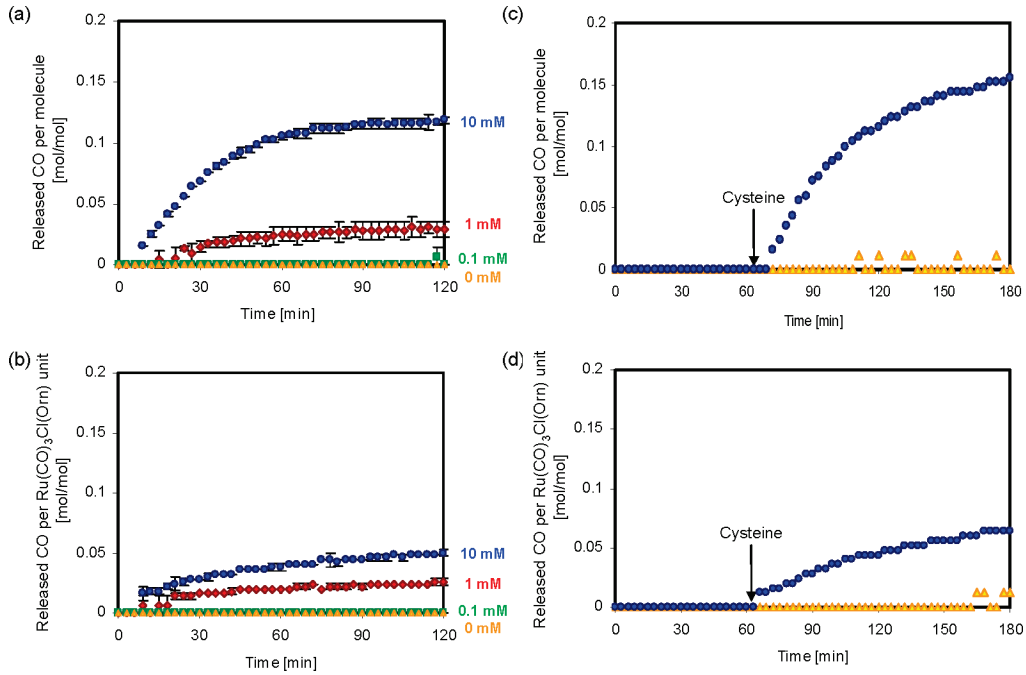

Figure 4. CO release of $Ru(CO)_3Cl$(glycinate) and the **7f** CO-releasing micelles as a function of time. CO release of (a) 1 mM $Ru(CO)_3Cl$(glycinate) and (b) 1 mM $Ru(CO)_3Cl$(ornithinate) moieties of the micelles in 50 mM PBS (pH7.4) in the presence of 10 (blue), 1 (red), 0.1 (green), and 0 (yellow) mM cysteine. (c) Stability of 1 mM $Ru(CO)_3Cl$(glycinate) in 80% FBS (yellow) and CO release upon addition of 10 mM cysteine after 60 min of incubation with 80% FBS (blue). (d) Stability of 1 mM $Ru(CO)_3Cl$(ornithinate) moieties of the micelles in 80% FBS (yellow) and CO release upon addition of 10 mM cysteine after 60 min of incubation with 80% FBS (blue).

$Ru(CO)_3Cl$(glycinate). This slow release may be due to the steric hindrance of the PEG corona, which hampers the diffusion of cysteine to the $Ru(CO)_3Cl$(ornithinate) moieties inside the micelles. We also assessed the effect of glutathione, another major thiol compound in the body, on the CO release of $Ru(CO)_3Cl$(glycinate) (Figure S13b). As was observed for cysteine, CO release was detected upon the addition of 10 mM glutathione. These data suggest that the compounds bearing thiol moieties are molecular cues to induce CO release from $Ru(CO)_3Cl$(glycinate) and micelles.

Despite the low concentration of cysteine (~8 μM) and glutathione (~2 μM) in human serum,[34,35] it is anticipated that the presence of other serum components may induce CO release or inactivate $Ru(CO)_3Cl$(glycinate) and the micelles. For this reason, we tested their stability in 80% fetal bovine serum (FBS). As can be seen in Figure 4c,d, both $Ru(CO)_3Cl$(glycinate) and the micelles did not liberate CO for 2 h and the amount of released CO was negligible even after 3 h. However, upon addition of 10 mM cysteine, CO release of both $Ru(CO)_3Cl$(glycinate) and the micelles was observed after incubation in serum for 1 h. Therefore, $Ru(CO)_3Cl$(glycinate) and the micelles are stable in serum and release CO when exposed to high concentrations of cysteine. This thiol-dependent CO release is interesting in terms of intracellular delivery of CO. It has been reported that a high concentration of cysteine exists in the endosome/lysosome compartment of mammalian cell lines,[36,37] whereas much lower cysteine levels are found in blood plasma. In addition, the cytoplasm contains glutathione levels in the millimolar range (0.5−10 mM).[38] Therefore, it is likely that $Ru(CO)_3Cl$(glycinate) and the micelles release CO primarily inside cells and to a lesser extent within the extracellular space.

Inhibition of LPS-Induced Inflammation. The anti-inflammatory effect of $Ru(CO)_3Cl$(glycinate) and the CO-releasing micelles was assessed using THP-1 Blue cells, which are derived from human monocyte THP-1 cells. This cell line is stably transfected with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter which is inducible by the pro-inflammatory transcription factor NF-κB. Upon the addition of LPS or other Toll-like receptor ligands, THP-1 Blue cells secrete SEAP via NF-κB activation. Thus, NF-κB activation can be assessed by measuring the SEAP levels in the medium. The cells were cultured in the medium containing $Ru(CO)_3Cl$(glycinate) or the CO-releasing micelles for 1 h followed by the addition of LPS (0.1 μg/mL). After 18 h of culture, the SEAP levels in the medium were analyzed. As shown in Figure 5a, the micelles suppressed the LPS-induced NF-κB activation in a dose-dependent manner. In particular, the addition of a 200 μM concentration (concentration of $Ru(CO)_3Cl$(ornithinate) moieties) of the micelles substantially suppressed the increase of the SEAP levels. Importantly, the addition of the micelles alone did not alter the SEAP levels. Since serum does not trigger CO release from the micelles (Figure 4d), this anti-inflammatory effect may only be attributed to CO release after cellular uptake of the micelles (see the Supporting Information, section S9). This release mechanism is presumably induced by cysteine in the endo/lysosome. In contrast, $Ru(CO)_3Cl$(glycinate) strongly increased the SEAP level after LPS stimulation. Surprisingly, the addition of $Ru(CO)_3Cl$(glycinate) alone also activated the NF-κB pathway. It is likely that the anti-inflammatory effects

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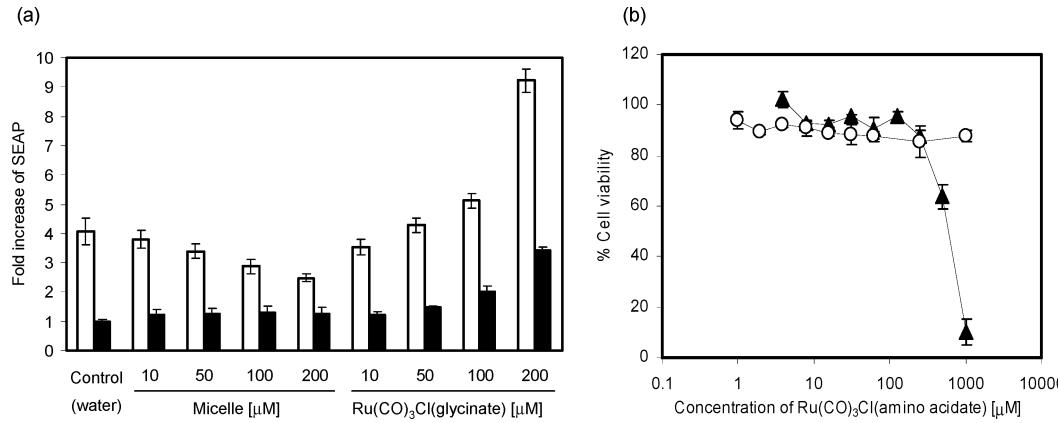

Figure 5. Biological activity of $Ru(CO)_3Cl$(glycinate) and CO-releasing micelles in vitro. (a) Anti-inflammatory effect of $Ru(CO)_3Cl$(glycinate) and CO-releasing micelles **7g**. THP-1 Blue cells were preincubated with $Ru(CO)_3Cl$(glycinate) or the micelles ($Ru(CO)_3Cl$(amino acidate) concentration 10, 50, 100, and 200 μM) for 1 h and cultured for 18 h in the presence (□) or absence (■) of 0.1 μg/mL LPS. The activation of the transcription factors was assessed by SEAP levels in the media. (b) Cytotoxicity of $Ru(CO)_3Cl$(glycinate) (▲) and the **7g** CO-releasing micelles (○). THP-1 Blue cells were incubated with $Ru(CO)_3Cl$(glycinate) or the micelles for 3 days. The cell viability was assessed by an MTT assay.

of $Ru(CO)_3Cl$(glycinate) were ablated by other side effects caused by the Ru compound, but the mechanims of these effects are unknown.[39]

It has been reported that inhalation of exogenous CO gas (250 ppm in the air supply) attenuates the LPS-induced NF-κB activation and the pro-inflammatory cytokine production of mouse macrophages and human monocytes.[5,40] However, treatment of human dendritic cells with 30 μM $[Ru(CO)_3Cl_2]_2$, another CO-releasing molecule, did not show any effect on the NF-κB activation despite the significant suppression of cytokine expression.[7] This confusing result of the CO-releasing molecule may be attributable to side effects of the Ru compound, although it is still possible that CO shows different actions depending on the cell type.

Since the NF-κB pathway is known to respond to diverse danger stimuli including cellular injury and stress signals,[41] we hypothesized that the strong NF-κB activation observed in the presence of $Ru(CO)_3Cl$(glycinate) was induced by its cytotoxicity. To demonstrate this, we performed an MTT assay for metabolic activity to assess cell viability in the presence of $Ru(CO)_3Cl$(glycinate) or the CO-releasing micelles. As expected, the viability of the cells decreased upon the addition of $Ru(CO)_3Cl$(glycinate) ($IC_{50} \approx 600$ μM); however, the micelles did not show any toxic effects up to 1 mM $Ru(CO)_3Cl$(ornithinate) moieties (Figure 5b). These data clearly show that $Ru(CO)_3Cl$(glycinate) or its byproducts after CO liberation are toxic to the THP-1 Blue cells and subsequently induced the NF-κB activation. In the case of the micelles, the toxic $Ru(CO)_3Cl$(ornithinate) moieties are hidden by a PEG corona. Thus, the stealth feature of PEG appears to inhibit unfavorable cellular responses.

Since the use of CO gas has potential risks in clinical applications, including the increase of carboxyhemoglobin levels, there is a need to develop a more efficient and convenient delivery system. The transition-metal carbonyl complexes have emerged as promising CO-releasing molecules to release known amounts of CO in response to biological stimuli. Nevertheless, as shown in this study, these compounds and/or their byproducts can have adverse effects. In contrast, CO-releasing micelles can deliver CO with much less toxicity. Therefore, the CO-releasing micelles could provide a safer CO-delivery system for immunotherapy as well as a useful platform for basic research to elucidate the physiological functions of CO.

In addition, it is of importance for immunotherapeutic applications to delivery of immunomodulatory drugs to lymph nodes, where a substantial fraction of immature dendritic cells and macrophages exists.[42] It has been well-established that particle size and surface chemistry are the most crucial factors for lymphatic uptake from the interstitial space.[43] We previously reported that PEGylated poly(propylene sulfide) nanoparticles in the range of 20−45 nm in diameter were efficiently delivered to the draining lymph nodes following intradermal injection.[24] Thus, the use of drug-delivery vehicles in this size range is a promising way to deliver drugs to lymph nodes without using any targeting ligands. The CO-releasing micelles are 30−40 nm in diameter, which is sufficiently small to allow lymph node targeting. Thus, CO-releasing species of colloidal dimensions, rather than low molecular weight CO-releasing molecules as amply described in the literature,[10−14] may be promising for CO release in lymph nodes draining an injection site for immunotherapy.

Conclusion

The use of CO for medical applications is challenging due to the difficulties in controlled delivery of this gas. To develop an administration method suitable for therapeutic applications, numerous interesting prodrugs of CO have been reported. Despite the successful control of CO-releasing properties by a chemical approach, rapid diffusion of these small molecules after administration remains a fundamental problem in controlled delivery for some applications, including ours, namely, targeting immune cells in lymph nodes that drain the injection site. We describe herein a novel approach to deliver CO using micelles

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as a CO donor. The amphiphilic PEG-*b*-OrnRu-*b*-nBu triblock copolymers formed monodisperse CO-releasing micelles in the range of 30−40 nm in diameter. CO release was induced by thiol compounds, including cysteine. The micelles successfully attenuated the LPS-induced inflammatory response of human monocytes. In addition, the toxicity of $Ru(CO)_3Cl$(amino acidate) moieties was significantly reduced by the stealth feature of PEG. These results show that the CO-releasing micelles can provide an efficient and safe administration method for CO-based therapy.

Acknowledgment. We thank Dr. Michael Groessl (Laboratory of Organometallic and Medicinal Chemistry, EPFL) for his help and advice in the ICP-OES experiment. We thank P. Schuck (NIH, Bethesda) for providing the SEDFIT software and B. Demeler (Health Science Center at San Antonio, University of Texas) for providing the UltraScan software and free access to the supercomputing resources through NSF Teragrid Science Gateway. We thank Dr. Karen Dane and Mr. Stephane Kontos (EPFL) for critical reading of the paper. This work has benefited from research funding from the European Community's Seventh Framework Programme via Project FP7-Health-2009 ENDOSTEM 241440 (activation of vasculature associated stem cells and muscle stem cells for the repair and maintenance of muscle tissue).

Supporting Information Available: Experimental Section, ^1H NMR spectra, GPC charts, IR spectra, colloidal stability of the micelles, UltraScan two-dimensional spectrum analysis (2DSA), Monte Carlo analysis (2DSA-MC), CO-release capacity of the micelles by the Mb assay, experimental setup for CO release measurement, CO release profiles in the presence of cysteine, glutathione, and $Na_2S_2O_4$, and cellular uptake of the micelles. This material is available free of charge via the Internet at http://pubs.acs.org.

JA1075025