Bio-reduction Hot Paper

DOI: 10.1002/ange.201404238

# Bio-reduction of Redox-Sensitive Albumin Conjugates in FcRn-Expressing Cells**

*Lorine Brülisauer, Gina Valentino, Sakura Morinaga, Kübra Cam, Jens Thostrup Bukrinski, Marc A. Gauthier, and Jean-Christophe Leroux**

***Abstract:*** *Disulfide-containing IgG-, Fc-, or albumin-based prodrugs that rely on FcRn-trafficking by endothelial cells for prolonged circulation in the body might be hampered by premature bio-reduction processes during FcRn-mediated recycling events. A detailed bio-reduction analysis of redox-sensitive albumin conjugates in two FcRn-expressing cell lines has been performed. The obtained results indicate that the FcRn-mediated recycling pathway is not (or is only poorly) bio-reducing.*

Serum albumin and proteins of the immunoglobulin G (IgG) family have exceptionally long circulation times in the body with a half-life of 19–23 days in humans.[1] This is due to their effective recycling mediated by the major histocompatibility complex class I-related Fc receptor (FcRn) expressed in endothelial cells and in organs such as the kidneys, liver, and intestine.[1,2] Albumin and IgG are taken up by these cells by macropinocytosis and subsequently bind to FcRn during endosomal acidification (< pH 6.5). FcRn then protects these proteins from progression to lysosomes for degradation by re-directing them to the extra-cellular space where release is triggered by pH neutralization.[3] From a biotechnological standpoint, this particularity enables IgG-based prodrugs (that is, antibody-drug conjugates) to circulate long enough to target specific locations in the body,[2b,4] and provides a means for increasing the circulation lifetime of small-molecule drugs in the form of Fc-[5] or albumin-based prodrugs.[6] The therapeutic potential of these prodrugs relies on timely release of the drug by a cleavable linker. One highly investigated strategy to reversibly conjugate drug molecules to either full IgG, its constant Fc region, or to albumin, is to use disulfide-containing linkers.[7] These prodrugs can respond to the various local redox micro-environments they encounter as they transit through the body and within sub-cellular spaces. In an ideal drug-delivery approach, these prodrugs should (for the most part) resist thiol–disulfide exchange in the extra-cellular space owing to the low abundance of thiol reducing agents.[8] At the targeted site, cell-surface interactions and internalization may expose prodrugs to significantly higher amounts of reducing agents and redox enzymes, which should promote drug release.[7a,9] Unfortunately, transit of intact long circulating prodrugs to their destination could potentially be hampered by the multiple FcRn-mediated recycling events it undergoes en route.[10] Furthermore, should FcRn biology contribute strongly to bio-reduction, prodrugs may also be predisposed to release their therapeutic cargo within endothelial cells (lining blood vessels), which may not necessarily be the targeted cell population. The bio-reducing capacity of FcRn-mediated trafficking pathways may have a decisive effect on the rate and location of drug release for these important classes of therapeutics. Unfortunately, to date, the extent of bio-reduction (and consequent drug release) occurring during FcRn-mediated trafficking has yet to be examined. Better characterizing bio-reduction in this pathway may provide new perspectives for understanding and optimizing the performance of drug delivery systems that rely on FcRn-mediated recycling for prolonged circulation in the body.

To shed some light on these questions, redox-responsive probes designed to undergo FcRn-mediated trafficking were prepared (Figure 1 a). As FcRn binds both Fc and albumin in a comparable pH-dependent manner, and despite the fact that FcRn biology remains to be fully elucidated, it is reasonable to expect that both molecules are trafficked in a comparable manner.[11] Human serum albumin was selected as scaffold for preparing the probes as it can be conveniently and site-selectively modified at $Cys^{34}$, which is a residue not involved in albumin-FcRn interactions.[12] Two bio-reducible probes consisting of peptide disulfide heterodimers with different thiol–disulfide exchange kinetics (**1** and **2**) were appended to $Cys^{34}$ with full retention of the native folded structure of albumin (Supporting Information, Figures S1–S4). The probes were designed to be poorly fluorescent in their intact state due to the presence of both a green boron dipyrromethene fluorophore (BODIPY) and a quencher

[*] L. Brülisauer, G. Valentino, S. Morinaga, K. Cam, Prof. Dr. J.-C. Leroux
Swiss Federal Institute of Technology Zurich (ETHZ), Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences
Vladimir-Prelog-Weg 1–5/10, 8093 Zurich (Switzerland)
E-mail: jleroux@ethz.ch
Prof. Dr. M. A. Gauthier
Institut National de la Recherche Scientifique (INRS), EMT Research Center
1650 boul. Lionel-Boulet, Varennes, J3X 1S2 (Canada)
Dr. J. Thostrup Bukrinski
Novozymes A/S
Krogshoejvej 36, 2880 Bagsvaerd (Denmark)

[**] This work was financially supported by Novozymes A/S. L.B. gratefully acknowledges a doctoral fellowship from the Scholarship Fund of the Swiss Chemical Industry (SSCI) (2-70882-08). C. Wu (peptide disulfide synthesis), M. Detmar and F. Roudnicky (HUVEC cell line), A. Helenius (RFP-Rab7 plasmids), the Scientific Center for Optical and Electron Microscopy (ETHZ), and the Functional Genomics Center (UZH) are gratefully acknowledged for their experimental support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201404238.

  

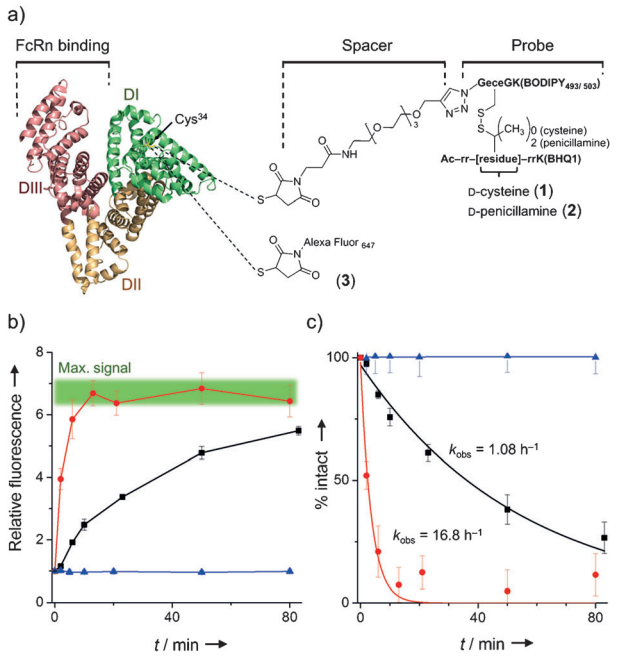


***Figure 1.*** Albumin-based probes for monitoring bio-reduction. a) Human serum albumin was site-selectively modified at $Cys^{34}$ (domain I; DI) with two redox-probes (**1** and **2**) or a fluorophore (**3**). This modification is away from the FcRn-binding domain III (DIII). Abbreviations: G glycine, e D-glutamic acid, c D-cysteine, K L-lysine, r D-arginine. b) In the presence of 1 mM glutathione (GSH, mimicking cytoplasm), pH 7.5 and 37 °C, **1** (●), and **2** (■) (500 nM) exhibit a circa 7-fold increase of fluorescence while **3** (▲) (500 nM) remains unaffected. c) Plotting the data from (b) as % intact probe vs. time with a 7-fold increase in fluorescence used as complete cleavage and using pseudo-first order kinetics analysis yields the observed rate constants for thiol–disulfide exchange with glutathione. Mean ± S.D. ($n = 3$).

(Black Hole Quencher, BHQ1) in the peptide disulfide system. (Bio-)reduction caused the departure of the peptide bearing BHQ1, leading to a circa 7-fold increase of fluorescence intensity, which was used for quantification (Figure 1b; Supporting Information, Figure S5). A short spacer was used to decouple disulfide exchange from any specific interactions with albumin, thus making findings more generalizable. Indeed, the observed rate constants for disulfide exchange of **1** and **2** in model redox buffers were consistent with those observed for comparable peptide disulfides not appended to albumin (Figure 1b).[13] **1** contains a sensitive Cys–Cys disulfide bond, while **2** possesses a Pen–Cys disulfide bond which has a circa 17-fold slower exchange rate (Figure 1c). These two types of disulfide bonds were selected to cover the range of disulfide exchange kinetics typically examined in prodrugs, thus permitting a more robust analysis of bio-reduction in the intra- and extra-cellular spaces.[7a,b,14] As expected, acidification below pH 6.5 (required for binding to FcRn)[11b] significantly slowed disulfide exchange (Supporting Information, Figure S5). A non-bio-reducible probe (**3**) was also prepared for comparison (Figure 1). Furthermore, the fluorophores in **1**–**3** did not demonstrate pH-dependent fluorescence output in the pH range 4–7.5 (Supporting Information, Figure S6) and, being constructed from D-amino acids, fluorescence de-quenching of **1** and **2** was insensitive to various proteases as well as cell lysates (thiols quenched; Supporting Information, Figures S7 and S8).

Two cell lines naturally expressing the FcRn receptor (also verified by immunostaining, Supporting Information, Figure S10) were examined herein: human epithelial colorectal adenocarcinoma cells (Caco-2) and human umbilical vein endothelial cells (HUVEC).[15] For these cells, albumin was internalized by macropinocytosis, which is consistent with FcRn trafficking (Supporting Information, Figure S11). Probes **1** and **2** were incubated with confluent cell monolayers and total (intra- and extra-cellular) fluorescence was monitored with time using a microplate reader (Figure 2b;

a)

| Experiment | Thiols contributing to bio-reduction: Extra-cellular | Cell surface | Stage of uptake: Early | Stage of uptake: Late | Cytosol |
|---|---|---|---|---|---|
| No pre-treatment ($k_1$) | + | + | + | + | + |
| Pulse–wash MPB ($k_2$) | + | | + | + | + |
| Co-incubation MPB ($k_3$) | | | | ± | + |
| $k_1 - k_2$ | | + | | | |
| $k_2 - k_3$ | + | | + | | |

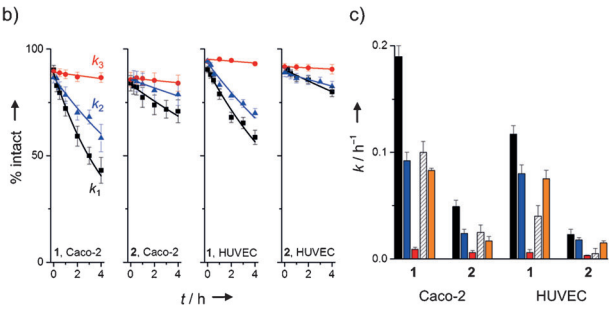


***Figure 2.*** Location-specific bio-reduction. a) Selective inhibition of thiol–disulfide exchange to deduce the relative contribution of thiols at different locations towards total bio-reduction. b) Kinetics of de-quenching of **1** and **2** (450 nM) in the presence of cells alone (■) or with thiol capping agent MPB (1 mM) (▲ pulse-wash; ● co-incubation) from which are extracted the observed de-quenching rate constants ($k_{1\text{-}3}$, pseudo first-order kinetic analysis). c) Observed (black: $k_1$, blue: $k_2$, red: $k_3$) and deduced (white: $k_1 - k_2$, orange: $k_2 - k_3$) rate constants of exchange of the probes indicating extra-cellular bio-reduction. Mean ± S.D. ($n = 6$–9).

Supporting Information, Figure S12). Addition of dithiothreitol, a cell-membrane permeable reducing agent, at the conclusion of the experiment led to the full recovery of the expected fluorescence. Thiol-insensitive **3** showed no change of fluorescence intensity over time in the presence of cells and lysosomal proteases were not involved in the recovery of fluorescence (Supporting Information, Figures S13 and S14). Thiols secreted by the cells into the extra-cellular space, thiols from the cell surface, from all recycling/uptake pathways, and from the cytosol can potentially contribute to bio-reduction. To better distinguish the relative contribution of these different thiols towards total bio-reduction, selective blocking experiments were performed (Figure 2a): a pulse-wash of the membrane impermeant thiol blocker *N*-(3-maleimidopropionyl)biocytin (MPB)[16] prior to addition of **1** or **2** selectively capped thiols on the cell-surface; co-incubation of MPB

  

blocked thiols in the extra-cellular space, on the cell surface, and within the early stages of endocytosis. By comparing the observed rate constants from these experiments ($k_{1\text{-}3}$, obtained by non-linear regression using a pseudo-first order kinetic model), the relative bio-reducing capacity of these different thiols can be distinguished (Figure 2a and c, numerical values in the Supporting Information, Table S1).

The observed rate constants for bio-reduction ($k_1$) decreased from **1** to **2** as expected due to the presence of the geminal methyl groups of the Pen–Cys disulfide on **2**. However, within a given cell line similar trends were observed with respect to the relative contribution of different locations towards bio-reduction of **1** and **2**. The major difference observed was between the cell lines themselves. The data indicate early uptake pathways and secreted thiols ($k_2$–$k_3$) as the dominant locations of bio-reduction for HUVEC cells (see below), while a more important contribution of the cell surface was observed for Caco-2 ($k_1$–$k_2$) in accordance with previous observations by our group made with a disulfide-bearing cationic dendrimer.[17] The small divergence between Caco-2 and HUVEC cells may result from the fact that, owing to the specific spreading characteristics, the number of HUVEC cells within the confluent monolayers examined (50000 per well) was significantly lower than for Caco-2 (270000 per well) and the other cell lines from the literature (160000 per well).[17]

By comparing the rate corresponding to early uptake pathways and secreted thiols ($k_2$–$k_3$), to the observed rates of de-quenching obtained with 1 mM glutathione in Figure 1b, the calculated average steady-state "GSH equivalent" concentration required to achieve comparable kinetics would be 9 μM and 11 μM for HUVEC and Caco-2, respectively (Supporting Information, Table S2). Despite the simplifications and assumptions made for obtaining these values, they nevertheless scale reasonable well with the actual measured concentration of thiols in the medium (17 μM and 9 μM at the 4 h time point, for HUVEC and Caco-2 cells, respectively). This suggests that, in this experimental setup for which the volume of the extra- cellular space is significantly larger than the intra-cellular one, the relative contribution of early uptake pathways to bio-reduction is low. Thus, bio-reduction ($k_1$) in Figure 2b shows the de-quenching of **1** and **2** in the extra-cellular space of the cells, with more-or-less proportional contributions from secreted thiols (likely glutathione) and thiols of the cell membrane (for example, protein disulfide isomerase).[7a,18] It is interesting to note that this result contrasts with the observation made for bio-reducible cationic dendrimers with Caco-2 cells: bio-reduction strongly involved the cell surface.[17] Indeed, while cationic dendrimers are expected to rapidly bind from solution to the negatively charged cell membrane, the albumin probes evaluated herein are likely to remain longer in solution, which may be at the origin of this difference. This finding highlights once again that extrapolation of conclusions drawn from bio-reduction experiments to other types of bio-reducible drug carriers is difficult. Overall, the observed rates of de-quenching of **1** and **2** for both cell lines suggest a strong involvement of the extra-cellular space in bio-reduction, which highlights the necessity of stabilizing disulfides to prevent premature drug release.

As **2** remained mostly intact over the 4 h period monitored in Figure 2b, this probe was to use to examine bio-reduction within the intra-cellular space by confocal fluorescence microscopy. Both Caco-2 and HUVEC cells were exposed to a pulse-wash of **2** and fluorescence was monitored with time over 3 h (Figure 3). Given that the fluorescence of **2** is quenched and thus invisible in its intact state, the cell membrane-permeant reducing agent glutathione monoethyl

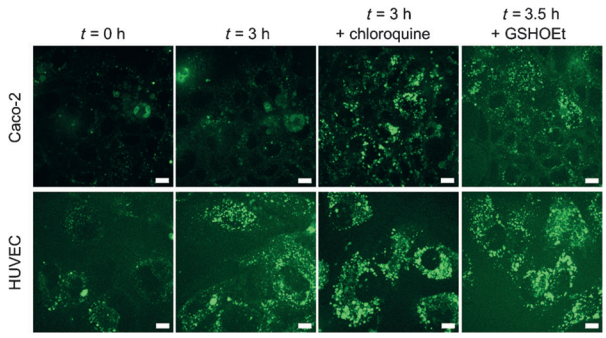


***Figure 3.*** Intra-cellular bio-reduction. Representative confocal fluorescence images of cells exposed to a 30 min pulse of **2** (1.35 μM), followed by a wash. Evolution of fluorescence was observed with time. To determine complete bio-reduction GSHOEt (38 mM) was added after the 3 h period. Co-incubation of cells, which have been exposed to **2**, with chloroquine (150 μM) was performed to disrupt endosomes. Scale bar: 10 μm.

ester (GSHOEt) was added after 3 h to access what the maximal possible cell signal at this time point would be. For the Caco-2 model, no evolution of fluorescence intensity of the image or number of fluorescent vesicles was observed within the timeframe evaluated (Figure 3). In contrast, almost full recovery of fluorescence of **2** was observed for HUVEC cells over a 3 h period. In both cases, co-incubation of cells (that have been exposed to **2**) with the endosomal pH buffer chloroquine over the 3 h period led to complete bio-reduction (Figure 3). This is in agreement with the expected endosomolytic behavior of chloroquine, which exposes endocytically entrapped **2** to cytoplasmic reducing agents and almost neutral pH. Indeed, the cytoplasm of both cell lines is shown to be sufficiently reducing to promote exchange of the sterically hindered Pen–Cys disulfide in **2**. As a negative control experiment, the thiol-insensitive **3** showed no evolution of fluorescence intensity in both cell lines as well as no effect of chloroquine or GSHOEt on fluorescence intensity (Supporting Information, Figure S15). Intra-cellular proteases were not involved in the recovery of fluorescence (Supporting Information, Figure S16).

To better understand the different intra-cellular bio-reduction behavior of Caco-2 versus HUVEC cells, co-localization of **2** with Rab7, a marker for late endosomes, over a 3 h period was performed. As seen in Figure 4a, in Caco-2 no co-localization between **2** and Rab7 was observed. Caco-2 had only a weak green background signal that did also not co-localize with Rab7. In contrast, substantial co-localization between **2** and Rab7 was observed for the HUVEC cells (Figure 4b). Nevertheless, HUVEC cells intrinsically

  

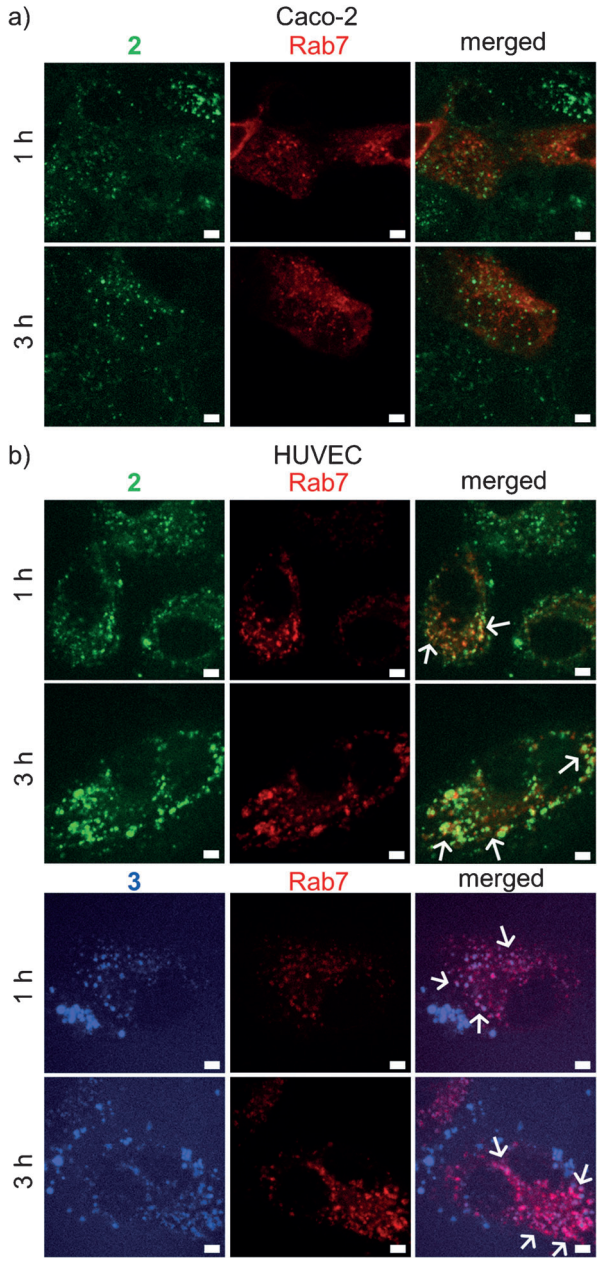


***Figure 4.*** Co-localization of albumin probes with Rab7. Representative live cell confocal images of: a) Caco-2 (expressing RFP-tagged Rab7) exposed to a 30 min pulse of **2** (1.35 μM), followed by a wash. Addition of GSHOEt (38 mM, 0.5 h), followed by a wash, reduced intra-cellular **2**. Cells were monitored with time and little or no co-localization between **2** and Rab7 was observed; b) HUVEC (expressing RFP-tagged Rab7) exposed to **2** and GSHOEt as described in (a) and HUVEC exposed to a 30 min pulse of **3** (5 μM), followed by a wash. Substantial co-localization between probes and Rab7 was observed suggesting a lysosomal pathway for albumin in HUVEC. Scale bar: 5 μm.

displayed a strong green background, with many green dots, which also co-localized with Rab7. Therefore, this result was confirmed by performing the analogous experiment using **3**, which fluoresces far-red rather than green (Figure 4b).

To further characterize intracellular trafficking, exocytosis was examined by flow cytometry on Caco-2 and HUVEC cells treated with a pulse-wash of **3**. The intensity of intra-cellular fluorescence of Caco-2 decreased progressively over 3 h, suggesting exocytosis of **3** in support for an FcRn-mediated recycling pathway (Supporting Information, Figure S17). Conversely, HUVEC cells displayed no change in cell fluorescence intensity over time, which is indicative of albumin processing in the lysosomal compartment. These combined results suggest that FcRn-mediated trafficking pathways are poorly bio-reducing, while intra-cellular bio-reduction of **2** in HUVEC cells is due to (at least) partial progression of albumin to lysosomes. Such a reducing capacity of degradative compartments could stem, for example, from the gamma-interferon-inducible lysosomal thiol reductase (GILT), which is found in late endosomes/lysosomes.[7a,19] Although FcRn expression in HUVEC was observed, the level of expression was significantly lower than for Caco-2 (Supporting Information, Figure S10). This decreased expression of the receptor could explain why albumin was less protected by FcRn from a degradative pathway in HUVEC cells (that is, owing to the saturation of the smaller receptor population) and, with that, from bio-reduction. It should be noted that non-polarized HUVEC cells were used in the experimental setup. Polarized cells, as are present in vivo, could have different FcRn expression/regulation. Thus, direct extrapolations of the findings in this study, beyond conceptual ones, to the in vivo environment should be made with great care owing to expected differences of FcRn recycling kinetics in the body.

In summary, results from this study corroborate findings from the literature that cell-secreted thiols and the cell surface are strongly involved in bio-reduction processes.[17,20] Bio-reduction in these areas will lead to non-specific release of the drug into the extra-cellular space. The results also suggest that the FcRn-mediated recycling pathway itself is not (or is poorly) bio-reducing. Thus, cells with an efficient, non-saturated FcRn recycling mechanism (that is, vascular FcRn system)[10,21] might be less susceptible to off-target effects from bio-reducible IgG-, Fc-, or albumin-prodrugs. However, should the FcRn-binding domain of these prodrugs be altered by for example, excessive grafting of drug molecules,[6a,22] behavior similar to that observed in the HUVEC cells may be observed (that is, impaired recycling leading to degradative pathways).[23] Overall, FcRn-mediated recycling increases the circulation lifetime of FcRn-binding prodrugs and protects from off-target bio-reduction resulting from macropinocytosis. These observations should be especially considered when the affinity between FcRn and the prodrug are manipulated to alter the pharmacokinetic profile of the latter.[12,24] Reduced affinity may not only result in shorter circulation times, but to higher proportionate release of drug in endothelial cells.

Received: April 11, 2014
Published online: June 24, 2014

**Keywords:** albumin · disulfide · drug delivery · FcRn receptors · reduction

[1] J. T. Andersen, I. Sandlie, *Drug Metab. Pharmacokinet.* **2009**, *24*, 318 – 332.
[2] a) T. T. Kuo, K. Baker, M. Yoshida, S.-W. Qiao, V. G. Aveson, W. I. Lencer, R. S. Blumberg, *J. Clin. Immunol.* **2010**, *30*, 777 – 789; b) D. C. Roopenian, S. Akilesh, *Nat. Rev. Immunol.* **2007**, *7*, 715 – 725.
[3] W. I. Lencer, R. S. Blumberg, *Trends Cell Biol.* **2005**, *15*, 5 – 9.
[4] R. Keizer, A. R. Huitema, J. M. Schellens, J. Beijnen, *Clin. Pharmacokinet.* **2010**, *49*, 493 – 507.
[5] T. Suzuki, A. Ishii-Watabe, M. Tada, T. Kobayashi, T. Kanayasu-Toyoda, T. Kawanishi, T. Yamaguchi, *J. Immunol.* **2010**, *184*, 1968 – 1976.
[6] a) D. Sleep, J. Cameron, L. R. Evans, *Biochim. Biophys. Acta Gen. Subj.* **2013**, *1830*, 5526 – 5534; b) B. Elsadek, F. Kratz, *J. Controlled Release* **2012**, *157*, 4 – 28; c) Y. Wu, S. Ihme, M. Feuring-Buske, S. L. Kuan, K. Eisele, M. Lamla, Y. Wang, C. Buske, T. Weil, *Adv. Healthcare Mater.* **2013**, *2*, 884 – 894.
[7] a) G. Saito, J. A. Swanson, K.-D. Lee, *Adv. Drug Delivery Rev.* **2003**, *55*, 199 – 215; b) R. V. J. Chari, M. L. Miller, W. C. Widdison, *Angew. Chem.* **2014**, *126*, 3872 – 3904; *Angew. Chem. Int. Ed.* **2014**, *53*, 3796 – 3827; c) A. M. Wu, P. D. Senter, *Nat. Biotechnol.* **2005**, *23*, 1137 – 1146; d) S. C. Alley, N. M. Okeley, P. D. Senter, *Curr. Opin. Chem. Biol.* **2010**, *14*, 529 – 537; e) S. Mura, J. Nicolas, P. Couvreur, *Nat. Mater.* **2013**, *12*, 991 – 1003.
[8] a) L. Turell, R. Radi, B. Alvarez, *Free Radical Biol. Med.* **2013**, *65*, 244 – 253; b) S. E. Moriarty-Craige, D. P. Jones, *Annu. Rev. Nutr.* **2004**, *24*, 481 – 509.
[9] Y.-M. Go, D. P. Jones, *Biochim. Biophys. Acta Gen. Subj.* **2008**, *1780*, 1273 – 1290.
[10] J. Kim, W. L. Hayton, J. M. Robinson, C. L. Anderson, *Clin. Immunol.* **2007**, *122*, 146 – 155.
[11] a) C. Chaudhury, S. Mehnaz, J. M. Robinson, W. L. Hayton, D. K. Pearl, D. C. Roopenian, C. L. Anderson, *J. Exp. Med.* **2003**, *197*, 315 – 322; b) C. Chaudhury, C. L. Brooks, D. C. Carter, J. M. Robinson, C. L. Anderson, *Biochemistry* **2006**, *45*, 4983 – 4990.
[12] J. T. Andersen, B. Dalhus, J. Cameron, M. B. Daba, A. Plumridge, L. Evans, S. O. Brennan, K. S. Gunnarsen, M. Bjoras, D. Sleep, I. Sandlie, *Nat. Commun.* **2012**, *3*, 610 – 618.
[13] a) C. Wu, S. Wang, L. Brülisauer, J.-C. Leroux, M. A. Gauthier, *Biomacromolecules* **2013**, *14*, 2383 – 2388; b) C. Wu, C. Belenda, J.-C. Leroux, M. A. Gauthier, *Chem. Eur. J.* **2011**, *17*, 10064 – 10070.
[14] a) B. A. Kellogg et al., *Bioconjugate Chem.* **2011**, *22*, 717 – 727; b) S. Bauhuber, C. Hozsa, M. Breunig, A. Göpferich, *Adv. Mater.* **2009**, *21*, 3286 – 3306.
[15] a) B. L. Dickinson, K. Badizadegan, Z. Wu, J. C. Ahouse, X. Zhu, N. E. Simister, R. S. Blumberg, W. I. Lencer, *J. Clin. Invest.* **1999**, *104*, 903 – 911; b) Y. Zhao, Y. Liu, Z. Chen, C. Korteweg, J. Gu, *J. Histochem. Cytochem.* **2011**, *59*, 474 – 488.
[16] E. A. Bayer, M. G. Zalis, M. Wilchek, *Anal. Biochem.* **1985**, *149*, 529 – 536.
[17] L. Brülisauer, N. Kathriner, M. Prenrecaj, M. A. Gauthier, J.-C. Leroux, *Angew. Chem.* **2012**, *124*, 12622 – 12626; *Angew. Chem. Int. Ed.* **2012**, *51*, 12454 – 12458.
[18] a) B. Wilkinson, H. F. Gilbert, *Biochim. Biophys. Acta Proteins Proteomics* **2004**, *1699*, 35 – 44; b) D. C. Sullivan, L. Huminiecki, J. W. Moore, J. J. Boyle, R. Poulsom, D. Creamer, J. Barker, R. Bicknell, *J. Biol. Chem.* **2003**, *278*, 47079 – 47088.
[19] U. T. Phan, B. Arunachalam, P. Cresswell, *J. Biol. Chem.* **2000**, *275*, 25907 – 25914.
[20] a) W. Sun, P. B. Davis, *J. Controlled Release* **2010**, *146*, 118 – 127; b) A. G. Torres, M. J. Gait, *Trends Biotechnol.* **2012**, *30*, 185 – 190; c) S. Aubry, F. Burlina, E. Dupont, D. Delaroche, A. Joliot, S. Lavielle, G. Chassaing, S. Sagan, *FASEB J.* **2009**, *23*, 2956 – 2967; d) D. Pezzoli, M. Zanda, R. Chiesa, G. Candiani, *J. Controlled Release* **2013**, *165*, 44 – 53.
[21] J. Kim, C. L. Bronson, W. L. Hayton, M. D. Radmacher, D. C. Roopenian, J. M. Robinson, C. L. Anderson, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *290*, G352 – G360.
[22] E. Neumann, E. Frei, D. Funk, M. D. Becker, H.-H. Schrenk, U. Müller-Ladner, C. Fiehn, *Expert Opin. Drug Delivery* **2010**, *7*, 915 – 925.
[23] E. S. Ward, J. Zhou, V. Ghetie, R. J. Ober, *Int. Immunol.* **2003**, *15*, 187 – 195.
[24] a) V. Kenanova et al., *Cancer Res.* **2005**, *65*, 622 – 631; b) V. Kenanova, T. Olafsen, L. E. Williams, N. H. Ruel, J. Longmate, P. J. Yazaki, J. E. Shively, D. Colcher, A. A. Raubitschek, A. M. Wu, *Cancer Res.* **2007**, *67*, 718 – 726; c) E. S. Ward, R. J. Ober in *Advances in Immunology, Vol. 103* (Ed.: F. W. Alt), Academic Press, **2009**, pp. 77 – 115; d) J. Zalevsky, A. K. Chamberlain, H. M. Horton, S. Karki, I. W. L. Leung, T. J. Sproule, G. A. Lazar, D. C. Roopenian, J. R. Desjarlais, *Nat. Biotechnol.* **2010**, *28*, 157 – 159.