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Nanoparticle Assembly of Surface-Modified Proteins

Matthias Fach, Lydia Radi, and Peter R. Wich*

Institut für Pharmazie und Biochemie, Johannes Gutenberg-Universität Mainz, Staudingerweg 5, 55128 Mainz, Germany

Supporting Information

ABSTRACT: Nature's biomaterials such as peptides and proteins represent a valuable source of highly defined macromolecules. Herein we developed a nanoparticle drug delivery system based on the assembly of surface-modified proteins that can be transferred into organic solvents and represent the structural material of the carrier system. The particles are prepared by an oil-in-water nanoemulsion technique without the need of additional denaturation or cross-linking steps for stabilization. We achieve the necessary lipophilic solubility switch of the protein material by high surface PEGylation under conservation of the native three-dimensional protein structure. This study focuses on lysozyme as model enzyme for the preparation of empty and doxorubicin-loaded nanoparticles with an average diameter of 100 nm. The particles are stable in physiological buffers and only release their therapeutic payload into cancer cells after a time-dependent cellular uptake. We also transferred this approach to various proteins, exemplifying the universal applicability of our new preparation method for protein-based nanoparticles.

Many synthetic drug delivery systems for therapeutic applications are very similar in size and shape to biological nanostructures, like exosomes or viruses. Polymer-based particles are especially interesting because they provide high flexibility in their design and can be easily chemically modified.[1] However, they often lack in biocompatibility and degradability. Alternatives are nature's biopolymers such as carbohydrates, polypeptides, and proteins. These biomaterials are readily accessible, structurally well-defined, and trigger in most cases only a low reaction of the immune system.[2]

In particular, proteins are attractive as material for the formation of nanoparticles. They have several advantages over synthetic polymers in aspects of biodegradability, immunogenicity, stability, and toxicity.[3] It is easy to functionalize the polypeptide backbone via a range of bioconjugation methods. Because of these unique properties, protein-based nanocarriers are promising candidates for the delivery of drugs.[4] A variety of proteins such as albumin,[5] collagen,[6] silk,[7] or elastin[8] have been utilized for the preparation of nanoparticles. Notably albumin is a popular choice as macromolecular carrier, based on its natural function as transport protein. A solvent-free formulation of the anticancer drug paclitaxel bound to albumin (Abraxane) forms nanoparticles with a size of 130 nm and is approved by the FDA for the treatment of metastatic breast cancer.[9]

The preparation of protein nanoparticles can be classified into three main techniques: emulsification,[10] desolvation,[11] and thermal gelation.[12] Other methods are spray drying[13] and the self-assembly in micelles.[5b,14] However, basically all methods include the dissolution or denaturation of the native protein structure into hydrophobic materials that can assemble into biohybrid materials.[14,15] In addition, these materials are often stabilized by an irreversible cross-linking step using glutaraldehyde[5c] or diisocyanate[5d] for the reaction between neighboring proteins. However, this modification and possible residual toxic cross-linker raised concerns about *in vivo* applications.[16]

Herein we present a versatile approach for the preparation of protein-based nanoparticles as delivery system for therapeutic payloads (Figure 1). We perform a lipophilic surface modification of lysozyme to enable a mild solvent evaporation technique for the particle formation. This emulsion-based preparation method requires no cross-linking, denaturation, or the use of additional surfactants to form stable nanoparticles. It is possible to successfully encapsulate and transport doxorubicin (DOX) as model drug to cancer cells. Different to previously reported approaches that also use proteins as particle material, we applied a mild surface modification, which renders the protein fully soluble in water-immiscible organic solvents while still preserving the initial native three-dimensional structure.

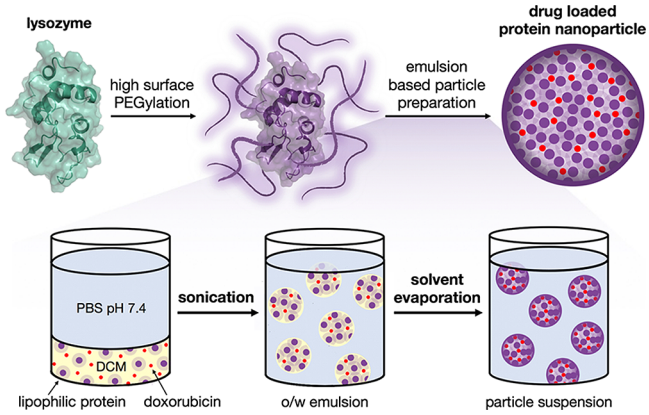

Figure 1. Emulsion-based nanoparticle preparation using surface-modified proteins as particle material. The native protein (green) gets highly PEGylated (purple) to increase its lipophilicity making it fully soluble in dichloromethane. Sonication forms an oil-in-water emulsion, followed by the evaporation of the organic solvent, which leads to a stable nanoparticle suspension without additional cross-linking. Therapeutic drugs like doxorubicin (red dots) can be encapsulated into the hydrophobic protein matrix.

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As shown with other biopolymers like nucleic acids[17] and polysaccharides,[18] it is possible to introduce hydrophobic moieties to obtain a solubility switch of the material. It is well documented that proteins can be stable in organic solvents without the denaturation of secondary structures.[19] Enzymes have been shown to even retain their catalytic activity, making them interesting for biotechnological applications in organic synthesis[20] and biocatalysis.[21] Among several methods, surface modification is most commonly used to increase protein stability and solubility in lipophilic environments like polymer thin films,[22] lipid membranes,[23] and organic solvents.[19] This can be achieved, for example, with the introduction of high amounts of polyethylene glycol (PEG). It is the gold standard for stealth polymers and represents the only clinical approved protein conjugate.[24] Even though it is not biodegradable and there are concerns about its immunogenicity,[25] PEG is generally regarded safe by the FDA. Currently, ten PEGylated proteins are already used for therapeutic applications.[26] Its hydrophilic nature allows reactions with proteins in aqueous solution.[27] At the same time the hydrophobic character enables modified enzymes to be soluble and active in organic solutions.[28] Inada et al. reported that various PEGylated enzymes become soluble and exhibit remarkable high activity in organic solvents.[29]

We used hen egg white lysozyme (LYZ) as model protein for our surface modification and the following nanoparticle (NP) preparation. LYZ is a bacteriolytic enzyme frequently found in nature[30] and human body fluids.[31] It has a catalytic center for cleavage of 1,4-β-linkages in peptidoglycan and chitodextrins, e.g., in bacterial cell walls.[32] Lysozyme is also used as food preservative, for pharmaceutical applications, and can inhibit tumor metastasis in mice.[33] The protein consists of 129 amino acids, has a total molecular weight of 14.3 kDa and a compact ellipsoid form (4.5 × 3.0 × 3.0 nm).[34]

To achieve a high PEGylation we attached multiple trichloro-*s*-triazine (TsT) activated methoxy-PEG chains (mPEG) to the protein surface. The TsT linker is highly reactive toward nucleophilic amino acids such as lysine, histidine, cysteine, and tyrosine.[35] This bioconjugation method is well studied, results in materials with minimal toxicity, and has proven successful in various therapeutic *in vitro* and *in vivo* applications.[36] While alternative linker strategies are preferred for site-specific and often mono-PEGylated protein conjugates, we benefit from the high TsT reactivity for the production of our lipophilic material. We were able to increase the molecular weight of the protein from 14.3 to 34.9 kDa by the attachment of 10 mPEG chains with an individual weight of 2 kDa (shown by SDS-PAGE, FPLC, and MALDI-ToF MS, see Figures S3–S5). The degree of functionalization corresponds with the number of available nucleophilic groups on the surface of lysozyme. Dynamic light scattering (DLS) shows an increase of the hydrodynamic diameter of lysozyme from 3.6 to 10.8 nm (Figure S6). This corresponds with transmission electron microscopy (TEM) images of LYZ(TsT-mPEG)$_{10}$ indicating a PEG corona around the protein core (see Figure S16). The structural integrity of the modified protein was analyzed by circular dichroism (CD, Figure S7 and Table S1). In addition we exposed the material to sonication conditions as used during particle preparation (Figure S8). In both cases no loss of secondary structure elements was observed. We also compared the activity of native lysozyme with the PEGylated form. The catalytic activity was lowered to 19% (Figure S9), which resembles similar reports of other protein modifications.[37] Together with the structural data, we conclude that the reduced activity most likely can be attributed to shielding effects of the dense surface PEGylation and possible modifications of amino acids close to the active site.

After PEGylation the protein is fully soluble in dichloromethane (DCM) without precipitation. The now predominant lipophilic character was confirmed by solvent extraction experiments (see Figure S11). Once in DCM the PEGylated protein LYZ(TsT-mPEG)$_{10}$ cannot be transferred back to the aqueous phase, even after vigorous mixing for 24 h. This is important for the following emulsion-based nanoparticle preparation. For this step LYZ(TsT-mPEG)$_{10}$ was dissolved in DCM and layered with a 5-fold excess of phosphate buffered saline (PBS). A sonication step creates an oil-in-water (o/w) emulsion. Interestingly, due to the amphiphilic character of PEG no additional surfactant is necessary to stabilize the nanoemulsion droplets. In the following step the volatile DCM evaporates, and the protein material in each droplet self-assembles into a particle matrix of tightly packed individual proteins. Now, the PEGylated proteins prefer the hydrophobic intermolecular interactions, and the particles, even empty ones, are stable without the need of additional cross-linking (Figure 2; Figures S15 and S17).

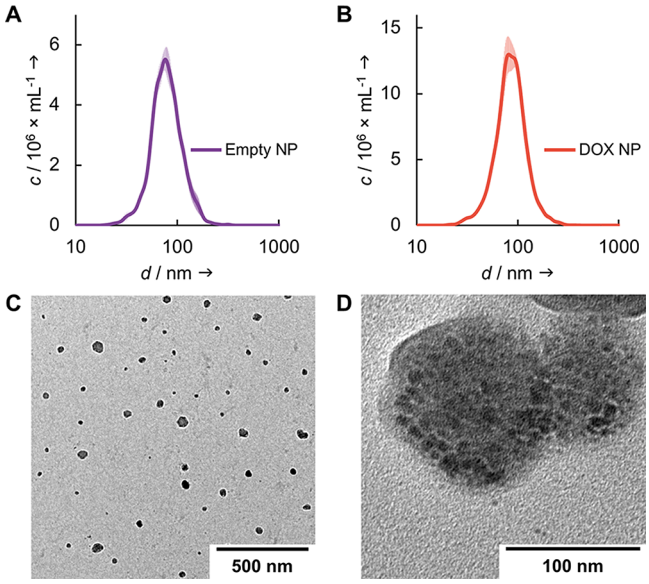

Figure 2. Nanoparticle tracking analysis results in a mean particle diameter of 94.6 nm for empty LYZ(TsT-mPEG)$_{10}$ particles (A) and 101.9 nm for DOX-loaded nanoparticles (B). TEM images of empty nanoparticles (overview (C) and magnification (D)) show the assembly of multiple proteins inside of single particles.

Interestingly, empty nanoparticles still show an enzymatic activity of 14% compared to the same amount of single PEGylated proteins (Figure S19). This decrease in activity can most likely be attributed to the effect that only lysozymes on the particle surface are accessible for substrates.

In addition to empty particles we encapsulated doxorubicin as hydrophobic model drug.[38] It represents a highly potent drug in the class of anthracyclines that are known to address a broad number of cancer types. Its cellular uptake is unspecific and leads to serious side effects like hematologic and cardiac toxicity.[39] Doxorubicin was added together with the modified proteins to the organic phase prior to the first emulsion step. During DCM evaporation the drug is physically entrapped in the particle matrix. Size measurements by nanoparticle tracking analysis (NTA) determined a mean diameter of 94.6 nm for empty

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particles and 101.9 nm for DOX-loaded particles (Figure 2A,B). Additional DLS measurements confirmed a hydrodynamic diameter of around 100 nm (Figure S15). The nanoparticle suspension is stable for several months without aggregation or precipitation. TEM measurements of empty particles confirm the assembly of multiple proteins into single particles (Figure 2D). The drug loading was determined by absorbance measurements at 488 nm resulting in a DOX concentration of 33 μM, with an entrapment efficiency of 9% and drug loading of 0.9 wt % (see section 3.7 in SI). The nanoparticles are stable in different physiological relevant buffer systems (at neutral pH) without leakage of the payload. A release was only detected under acidic conditions, in the presence of proteases or a reductive environment similar to the cytosol of cells (Figure S20).

We followed the cellular uptake of DOX-loaded nanoparticles by confocal laser scanning microscopy (CLSM) and analyzed the drug delivery. In order to monitor the fate of the particle material independent from the red fluorescent payload, we additionally labeled free acids on the surface of the PEGylated proteins with 6-aminofluorescein (F_LYZ(TsT-mPEG)$_{10}$, see section 2.2 in SI). We incubated these DOX-loaded and fluorescence-labeled nanoparticles (drug concentration: 4 μM) with cervical cancer cells (HeLa) and monitored the cellular uptake over time (Figure 3).

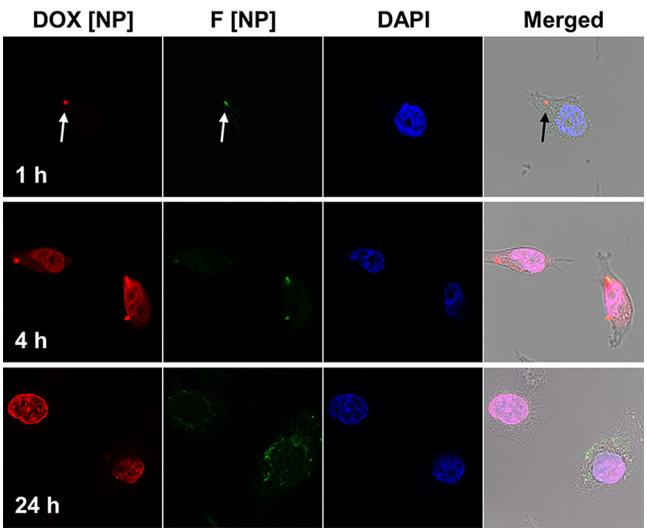

Figure 3. Time-dependent cellular uptake of doxorubicin, encapsulated in fluorescent-labeled nanoparticles. The particles are readily taken up by the cell within 1 h. After 4 h, endosomal release of red DOX into the cytosol can be observed. Released DOX finally accumulates in the nucleus after 24 h, whereas the fluorescent protein material is spread over the cytosol of the cell. Individual channels from left to right: red DOX signal (DOX [NP]), green fluorescence signal (F [NP]), blue core staining (DAPI), and a merged image of all channels with additional transmitted light images.

Confocal microscopy revealed that DOX-loaded lysozyme nanoparticles exhibit a time-dependent cellular uptake. After 1 h the particles concentrate in intracellular compartments. At this point both the red DOX signal and the green fluorescence of the particle material (F_LYZ(TsT-mPEG)$_{10}$) is colocalized. This shows that the nanoparticles are still fully assembled when taken up by the cells, presumably via an endosomal uptake mechanism. After 4 h, we observe a release of doxorubicin into the cytosol and the start of accumulation in the nucleus. Finally, after 24 h of incubation all doxorubicin can be found in the nucleus of the cell, whereas the green signal of the particle material is spread over the cytosol of the cell. This time-dependent uptake of DOX-loaded NPs observed by CLSM was additionally confirmed by flow-cytometry (Figure S23). Once taken up by cells, the encapsulated drug is most likely released due to the disassembly of the nanoparticles by proteases and changes of protein integrity under the reductive intracellular conditions. In comparison, free DOX × HCl enters the cell rapidly and uncontrolled by passive diffusion through the membrane, and fully colocalizes in the nucleus of the cell already after 1 h (Figure S25).

The successful delivery of DOX is reflected by the test on cell viability. Despite the different uptake mechanism, DOX-loaded nanoparticles and free DOX × HCl show similar therapeutic effects in concentrations up to 10 μM (see toxicity test, Figure 4 and Figure S22). In comparison, both the protein material itself and empty nanoparticles show no toxicity.

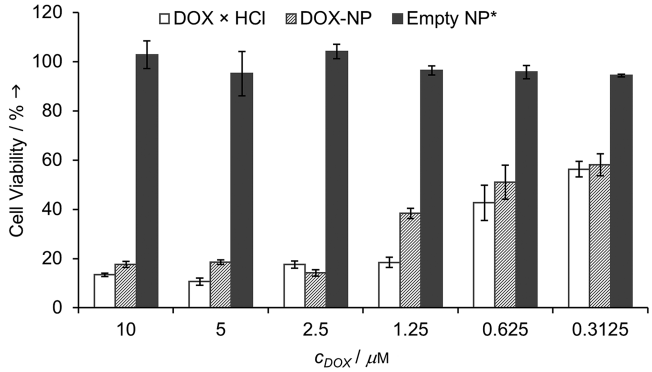

Figure 4. MTT toxicity assay with HeLa cells after 48 h. Free DOX × HCl (white bars) and DOX-loaded nanoparticles (striped) show comparable dose-dependent toxicity. Empty nanoparticles have no effect on cell viability (dark gray; *diluted in the same ratio as DOX-loaded nanoparticles to achieve the same particle concentration).

We were also able to transfer our nanoparticle preparation method to various proteins with molecular weights up to 67 kDa. Analogous to the procedure described above, we performed a high surface PEGylation resulting in lipophilic materials. The emulsion-based protein assembly results in nanoparticles with mean diameters from 94.6 to 207.7 nm (Table 1; Table S3 and Figure S26).

Table 1. Nanoparticles Prepared from Various Proteins

protein	mol wt (kDa)	particle diameter (nm)
lysozyme	14.3	94.6 ± 1.9
β-lactoglobulin	18.3	207.7 ± 2.9
ovalbumin	43.1	173.3 ± 5.9
human serum albumin	66.5	178.8 ± 5.3

In summary, we present a new concept for the preparation of protein-based drug delivery systems. The nanoparticles are formed in an emulsification process that entraps therapeutic payloads between lipophilic-modified protein building blocks. Furthermore, our method preserves the initial structure of the protein and does not require additional cross-linking to form stable particles. We were able to demonstrate in our model system that lysozyme-based nanoparticles show a controlled cellular uptake and can successfully deliver doxorubicin into cancer cells without loss of its therapeutic activity.

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Unlike the only commercially available protein-based drug delivery system (Abraxane), we are not limited to serum albumins as particle material. Our new approach for particle preparation has the potential to be universally applied to any protein or enzyme of choice. This will extend the range of possible biopolymer building blocks for the formation of protein nanoparticles considerably and open up new technological and pharmaceutical innovations for the delivery of drugs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06243.

Activation of mPEG; protein modification; particle preparation; cell culture (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*wich@uni-mainz.de

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sapsford, K. E.; Algar, W. R.; Berti, L.; Gemmill, K. B.; Casey, B. J.; Oh, E.; Stewart, M. H.; Medintz, I. L. *Chem. Rev.* **2013**, *113*, 1904.

(2) Nitta, S. K.; Numata, K. *Int. J. Mol. Sci.* **2013**, *14*, 1629.

(3) (a) Jutz, G.; Boker, A. *Polymer* **2011**, *52*, 211. (b) Witus, L. S.; Francis, M. B. *Acc. Chem. Res.* **2011**, *44*, 774.

(4) (a) Elzoghby, A. O.; Samy, W. M.; Elgindy, N. A. *J. Controlled Release* **2012**, *161*, 38. (b) Wu, Y.; Ng, D. Y.; Kuan, S. L.; Weil, T. *Biomater. Sci.* **2015**, *3*, 214.

(5) (a) Boyer, C.; Bulmus, V.; Liu, J.; Davis, T. P.; Stenzel, M. H.; Barner-Kowollik, C. *J. Am. Chem. Soc.* **2007**, *129*, 7145. (b) Xu, R.; Fisher, M.; Juliano, R. L. *Bioconjugate Chem.* **2011**, *22*, 870. (c) Langer, K.; Anhorn, M. G.; Steinhauser, I.; Dreis, S.; Celebi, D.; Schrickel, N.; Faust, S.; Vogel, V. *Int. J. Pharm.* **2008**, *347*, 109. (d) Piradashvili, K.; Fichter, M.; Mohr, K.; Gehring, S.; Wurm, F. R.; Landfester, K. *Biomacromolecules* **2015**, *16*, 815. (e) Elzoghby, A. O.; Samy, W. M.; Elgindy, N. A. *J. Controlled Release* **2012**, *157*, 168.

(6) Papi, M.; Palmieri, V.; Maulucci, G.; Arcovito, G.; Greco, E.; Quintiliani, G.; Fraziano, M.; De Spirito, M. *J. Nanopart. Res.* **2011**, *13*, 6141.

(7) Numata, K.; Kaplan, D. L. *Adv. Drug Delivery Rev.* **2010**, *62*, 1497.

(8) Bessa, P. C.; Machado, R.; Nürnberger, S.; Dopler, D.; Banerjee, A.; Cunha, A. M.; Rodríguez-Cabello, J. C.; Redl, H.; van Griensven, M.; Reis, R. L.; Casal, M. *J. Controlled Release* **2010**, *142*, 312.

(9) Green, M. R.; Manikhas, G. M.; Orlov, S.; Afanasyev, B.; Makhson, a. M.; Bhar, P.; Hawkins, M. J. *Ann. Oncol.* **2006**, *17*, 1263.

(10) Patil, G. V. *Drug Dev. Res.* **2003**, *58*, 219.

(11) Weber, C.; Coester, C.; Kreuter, J.; Langer, K. *Int. J. Pharm.* **2000**, *194*, 91.

(12) Cheng, Q.; Benson, D. R.; Rivera, M.; Kuczera, K. *Biopolymers* **2006**, *83*, 297.

(13) Lee, S. H.; Heng, D.; Ng, W. K.; Chan, H.-K.; Tan, R. B. H. *Int. J. Pharm.* **2011**, *403*, 192.

(14) Wu, Y.; Shih, E. K.; Ramanathan, A.; Vasudevan, S.; Weil, T. *Biointerphases* **2012**, *7*, 1.

(15) Wu, Y.; Wang, T.; Ng, D. Y.; Weil, T. *Macromol. Rapid Commun.* **2012**, *33*, 1474.

(16) (a) Han, B.; Jaurequi, J.; Tang, B. W.; Nimni, M. E. *J. Biomed. Mater. Res.* **2003**, *65*, 118. (b) Bolognesi, C.; Baur, X.; Marczynski, B.; Norppa, H.; Sepai, O.; Sabbioni, G. *Crit. Rev. Toxicol.* **2001**, *31*, 737.

(17) Abe, H.; Abe, N.; Shibata, A.; Ito, K.; Tanaka, Y.; Ito, M.; Saneyoshi, H.; Shuto, S.; Ito, Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 6475.

(18) (a) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Dashe, J.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2008**, *130*, 10494. (b) Wich, P. R.; Fréchet, J. M. J. *Aust. J. Chem.* **2012**, *65*, 15.

(19) Stepankova, V.; Bidmanova, S.; Koudelakova, T.; Prokop, Z.; Chaloupkova, R.; Damborsky, J. *ACS Catal.* **2013**, *3*, 2823.

(20) Koeller, K. M.; Wong, C. H. *Nature* **2001**, *409*, 232.

(21) (a) Illanes, A.; Cauerhff, A.; Wilson, L.; Castro, G. R. *Bioresour. Technol.* **2012**, *115*, 48. (b) Klibanov, A. M. *Nature* **2001**, *409*, 241.

(22) Sengonul, M.; Ruzicka, J.; Attygalle, A. B.; Libera, M. *Polymer* **2007**, *48*, 3632.

(23) Grogan, M. J.; Kaizuka, Y.; Conrad, R. M.; Groves, J. T.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2005**, *127*, 14383.

(24) (a) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chem., Int. Ed.* **2010**, *49*, 6288. (b) Kolate, A.; Baradia, D.; Patil, S.; Vhora, I.; Kore, G.; Misra, A. *J. Controlled Release* **2014**, *192*, 67. (c) Herzberger, J.; Niederer, K.; Pohlit, H.; Seiwert, J.; Worm, M.; Wurm, F. R.; Frey, H. *Chem. Rev.* **2016**, *116*, 2170.

(25) (a) Schellekens, H.; Hennink, W. E.; Brinks, V. *Pharm. Res.* **2013**, *30*, 1729. (b) Verhoef, J. J. F.; Anchordoquy, T. J. *Drug Delivery Transl. Res.* **2013**, *3*, 499.

(26) Pelegri-O'Day, E. M.; Lin, E. W.; Maynard, H. D. *J. Am. Chem. Soc.* **2014**, *136*, 14323.

(27) Pfister, D.; Morbidelli, M. *J. Controlled Release* **2014**, *180*, 134.

(28) (a) Muller, E.; Josic, D.; Schroder, T.; Moosmann, A. *J. Chromatogr. A* **2010**, *1217*, 4696. (b) Mayolo-Deloisa, K.; Lienqueo, M. E.; Andrews, B.; Rito-Palomares, M.; Asenjo, J. A. *J. Chromatogr. A* **2012**, *1242*, 11.

(29) (a) Inada, Y.; Takahashi, K.; Yoshimoto, T.; Ajima, A.; Matsushima, A.; Saito, Y. *Trends Biotechnol.* **1986**, *4*, 190. (b) Inada, Y.; Furukawa, M.; Sasaki, H.; Kodera, Y.; Hiroto, M.; Nishimura, H.; Matsushima, A. *Trends Biotechnol.* **1995**, *13*, 86.

(30) Callewaert, L.; Michiels, C. W. *J. Biosci.* **2010**, *35*, 127.

(31) Hankiewicz, J.; Swierczek, E. *Clin. Chim. Acta* **1974**, *57*, 205.

(32) Salton, M. R. *Bacteriol. Rev.* **1957**, *21*, 82.

(33) (a) Liburdi, K.; Benucci, I.; Esti, M. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 1062. (b) Mine, Y. *Curr. Pharm. Des.* **2007**, *13*, 875. (c) Yang, Y.; Hamaguchi, K. *J. Biochem.* **1980**, *87*, 1003. (d) Cocchietto, M.; Zorzin, L.; Veronesi, P. A.; Sava, G. *Mol. Med. Rep.* **2008**, *1*, 847.

(34) Blake, C. C. F.; Koenig, D. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, V. R. *Nature* **1965**, *206*, 757.

(35) (a) Abuchowski, A.; van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3578. (b) Wongpinyochit, T.; Uhlmann, P.; Urquhart, A. J.; Seib, F. P. *Biomacromolecules* **2015**, *16*, 3712.

(36) (a) Lim, J.; Simanek, E. E. *Adv. Drug Delivery Rev.* **2012**, *64*, 826. (b) Blackall, D. P.; Armstrong, J. K.; Meiselman, H. J.; Fisher, T. C. *Blood* **2001**, *97*, 551. (c) Banerjee, R.; Pace, N. J.; Brown, D. R.; Weerapana, E. *J. Am. Chem. Soc.* **2013**, *135*, 2497.

(37) (a) Mabrouk, P. A. *Bioconjugate Chem.* **1994**, *5*, 236. (b) Falatach, R.; McGlone, C.; Al-Abdul-Wahid, M. S.; Averick, S.; Page, R. C.; Berberich, J. A.; Konkolewicz, D. *Chem. Commun.* **2015**, *51*, 5343.

(38) Tacar, O.; Sriamornsak, P.; Dass, C. R. *J. Pharm. Pharmacol.* **2013**, *65*, 157.

(39) (a) Volkova, M.; Russell, R. *Curr. Cardiol. Rev.* **2011**, *7*, 214. (b) Hortobágyi, G. N. *Drugs* **1997**, *54*, 1.

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