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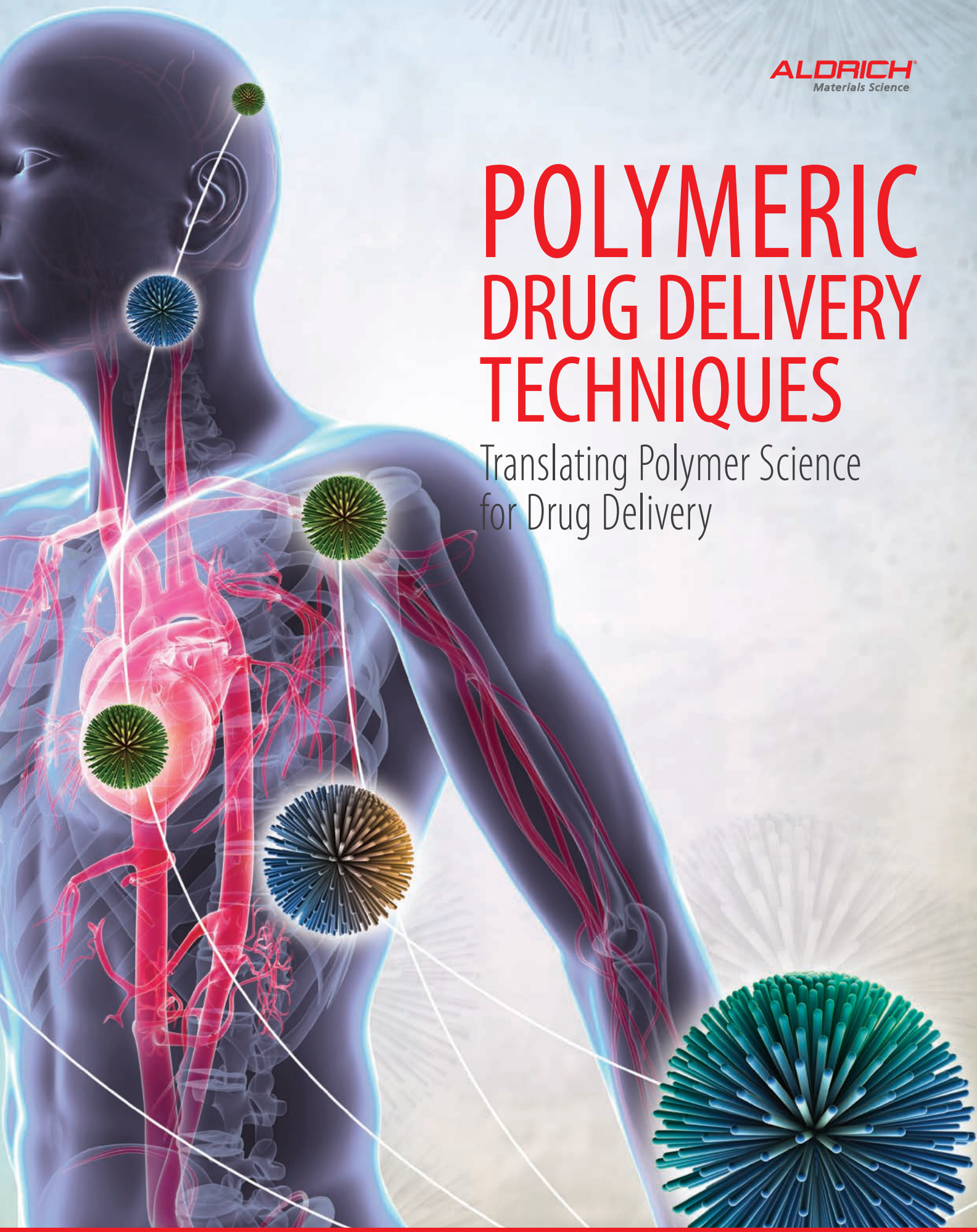
POLYMERIC DRUG DELIVERY TECHNIQUES

Translating Polymer Science for Drug Delivery



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Preface

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Rapid advances in medicine and biotechnology have driven the field of drug discovery and led to the development of many new highly potent and target-specific drug candidates. Despite the fast pace of research and early-stage discovery, many drug candidates fail during preclinical evaluation due to poor efficacy, limited bioavailability, and other challenges associated with effective drug delivery. Small molecule drugs can suffer from low solubility, poor stability, short circulation time, and non-specific toxicity limiting their therapeutic efficacy. Biopharmaceuticals such as nucleic acids, peptides, and proteins are often limited by poor stability and rapid clearance from the body. These challenges, coupled with the complexity and diversity of new pharmaceuticals, are fueling the evolution of novel drug delivery systems that overcome bioavailability and delivery obstacles. However, despite the growing importance of polymer drug delivery methodologies, the materials and methods of drug delivery are not widely available to those outside the polymer synthesis field.

The objective of effective drug delivery is improving the pharmacokinetics and pharmacodynamics of each therapeutic to enable drug delivery to the right place, at the right time and in the right amount. Delivery systems apply three main strategies to enable improved drug efficacy.

Controlled Release

Drug efficacy can be enhanced by maintaining the concentration within the therapeutic window (effective dose). Polymer carriers loaded with therapeutics enable controlled temporal and spatial release of a drug by controlling drug diffusion, the rate of dissolution, or degradation of the carrier.

Targeted Delivery

Drug efficacy can be enhanced and toxicity minimized by localization at the organ, tissue, cellular, or organelle level. Targeting can be achieved by coating or conjugating the carrier with affinity reagents such as nucleic acids, peptides, antibodies, or others that bind specific cell receptor proteins, nucleic acids, or polysaccharides.

Solubility Enhancement

Low drug solubility and stability often reduce the effectiveness of an otherwise promising therapeutic candidate. Drug delivery systems can be formulated to improve the *in vivo* solubility of lipophilic and hydrophobic drugs by encapsulation in a drug delivery carrier or by conjugation with a polymer.

Selecting a Polymeric Drug Delivery System

There are three main categories of polymeric drug delivery systems; colloidal carriers (micro, nanoparticles, micelles, micro/nanogels), implantable networks or hydrogels, and polymer drug conjugates. Unfortunately, there is no "silver bullet" for effective delivery of broad classes of therapeutics. Rather, selection of a drug delivery system must be driven by the nature of the drug and the inherent properties of the drug delivery system (**Figure 1**). Drug properties, including chemistry, solubility, potency, site of action, and clearance rate, each impact the proper selection of a drug delivery system that can achieve the desired outcomes. In addition, the choice of drug delivery system determines the drug loading capacity, longevity of release, and the route best suited for administration. Furthermore, characteristics of the drug delivery system (size, surface charge and hydrophobicity, shape, flexibility, inclusion of targeting moieties) will affect performance and distribution in the body. Each drug delivery system has inherent advantages and limitations (**Table 1**). It should be noted that drug release from any carrier is determined by a complex interaction between the drug properties, polymer characteristics, and environmental/*in vivo* conditions.

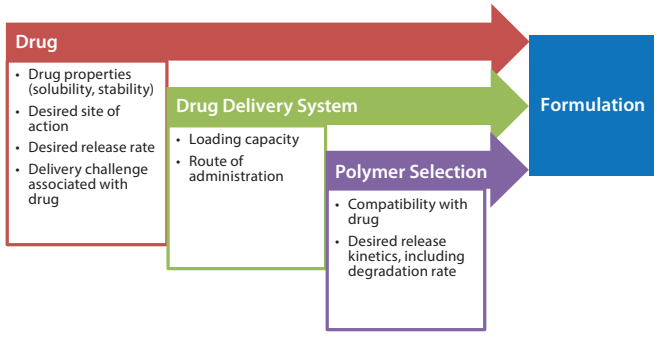

Figure 1. Drug delivery formulation selection process.

Table 1. Advantages and limitations of drug delivery systems.

Drug Delivery System and Polymer Types	Advantages	Limitations
Microparticles		
• Biodegradable polymers • Natural polymers	• Encapsulate a variety of drugs • Sustained release can be achieved	• Burst release possible, may lead to local toxicity
Nanoparticles		
• Biodegradable polymers • Natural polymers	• Stable delivery system • Small size enables enhanced retention and permeation into tissue and tumor	• Non-specific uptake in RES
Micelles		
• Amphiphilic block copolymers	• Enhanced solubility for hydrophobic drugs • Facile synthesis	• Less stable, may require additional crosslinking
Drug Conjugates		
• Hydrophilic polymers • Dendrimers	• Extended circulation half-life, reduced clearance due to increased drug hydrodynamic radius • Decreased drug immunogenicity and degradation	• Activity of drug can decrease due to conjugation • Approach provides sustained but not controlled release • Low loading capacity of drug
Hydrogels or Implants		
• Hydrophilic polymers • Biodegradable polymers • Natural polymers	• Broad range of release timeframes (weeks to months) • Useful for localized delivery • Improved patient compliance due to infrequent dosing	• Drug solubility may limit utility • Limitation to route of delivery achievable • Delivery may require incision or larger gauge needle • Risk of local dose dumping

Drug Delivery Material Choices

Polymer selection greatly influences the performance of the drug delivery system. Careful polymer selection is essential to control the encapsulation efficiency, release rate, and duration of release. Many polymers can be formulated into various drug delivery systems to address the three key drug delivery strategies to enable improved drug efficacy (**Table 2**). The diversity of polymer building blocks can further complicate formulation decisions. As discussed by Du and Stenzel (in this publication), the most critical factor in polymer selection is considering the interaction of the drug and polymer. Polymer selection will determine the mechanism for drug release (bulk erosion, system degradation), and the choice of polymer properties (molecular weight, surface charge) will influence release rate and impact pharmacokinetics. Further fine-tuning of release from drug delivery systems can be achieved by using multiple types of polymers or including additives.

Table 2. Polymer categories and the drug delivery strategies they enable.

	Controlled Release	Targeted Delivery	Solubility Enhancement
Biodegradable nanoparticles	✔	✔	
Biodegradable micelles			✔
Responsive polymers	✔	✔	
Polymeric hydrogels	✔	✔	
PEG conjugation			✔
Polyoxazoline polymers			✔
Dendrimers		✔	✔

About This Guide

This guide is intended to provide an overview of polymeric drug delivery systems as well as provide the corresponding example formulation protocols and product information required to utilize these techniques in the laboratory. The publication has been developed to enable those without a polymer chemistry background to use polymers to solve their drug delivery research challenges; but, we have also kept the expert in mind by including a number of cutting-edge methodologies. This guide is arranged according to drug delivery strategies, and these strategies are noted within each method. We hope this publication will enable chemists, engineers, pharmaceutical scientists, and biologists to explore different drug delivery techniques to facilitate translational research.

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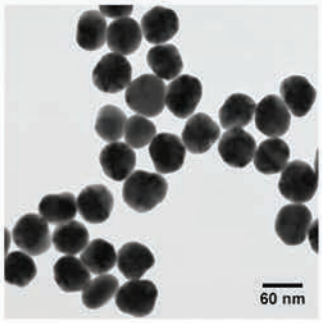

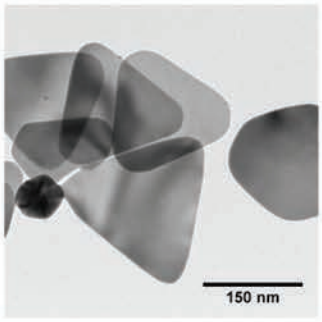

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POLYMER MICELLES FOR DRUG DELIVERY


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Introduction

Polymeric micelles obtained from the self-assembly of amphiphilic block copolymers are probably one of the most common drug delivery carriers among polymeric nanoparticles.[1–4] The rise of highly controlled polymerization techniques, especially processes such as ATRP[5] and RAFT,[6] has led to an extraordinary surge of new types of block copolymers fit for biomedical applications. Facile control over the polymer structure has also meant access to a large array of self-assembled morphologies including micelles, cylindrical micelles, and polymersomes. Micelles in particular are at the center of attention as potential drug carriers due to a core-shell structure that is highly water soluble while still maintaining a hydrophobic core suitable for hydrophobic drugs. This is crucial for many drugs since they are often rendered insoluble in water, and loading them into drug carriers can increase their solubility by several orders of magnitude.[4,7]

Choice of Block Copolymers

The choice of drug carriers can be daunting. In addition to a range of commercially available block copolymers, there is basically no limit to the design of amphiphilic structures thanks to advances in polymer design. Block copolymers can be further complemented by other amphiphilic polymers (such as miktoarm starpolymers, multiblock copolymers, and star polymers) to enable the formation of compartmentalized micelles. Whatever architecture is chosen, the primary consideration should be the compatibility between the drug and the polymers.[7] The polymer–drug interaction plays an important role in the drug-loading capacity of a carrier and the stability of the drug in the matrix, which ultimately affects the shelf-life of the carrier. The miscibility of a drug with the polymeric matrix can be described by the Flory–Huggins theory. This contains both entropy and enthalpy components, expressed by the Flory–Huggins interaction parameter χ, that describe the interaction between the polymer and the drug. In other words, the Flory–Huggins parameter χ is a measure of compatibility between polymer and drug.

Since many drugs have a strong tendency to crystallize, theoretical models of the polymer–drug interactions treat this like a solution where the presence of the homogenous mixture is determined by the miscibility curve of its phase diagram on the molecular level. Moreover, the models discussing the thermodynamic stability of a binary system are based on a fast equilibrium. This may not always be the case since polymers with high T_g values may trap the drug in the matrix, resulting in a kinetically stable system. Readers who are interested in the underpinning thermodynamic principles are referred to an excellent review article.[8]

How, then, can one choose the right polymer for the right drug to achieve good loading and high stability? The assumption "like dissolves like" is a good starting point. This rule of thumb is based on the Flory–Huggins parameter χ in **Equation 1**:

$$\chi = \frac{(\delta_s - \delta_p)^2 v_s}{RT} \qquad (1)$$

where δ_s and δ_p are the Scatchard–Hildebrand solubility parameter of the solute and the polymer, respectively.[7] In short, polymers that are chemically similar to the drug should enable the highest loading capacity. A good example is doxorubixin conjugated to a polymer.[9] While the drug attached to the polymer was found to be inactive, polymer micelles constructed with the polymer-drug conjugate created an environment that had the highest compatibility possible with free doxorubicin leading to an increased loading capacity. Decorating the polymer with the same drug to be loaded is an effective but cost-prohibitive option. Alternatively, subtle changes to the interior polymeric structure by altering the substitution of the polymer can maximize loading. For example, a PEO-b-PCL polymer was modified with benzyl, carboxyl, stearyl, palmitoyl, and cholesteryl functional groups with the aim of varying the hydrophobicity to tailor the polymer matrix toward the highest possible loading capacity of the chosen drug.[10]

However, not every lab has synthetic chemists capable of carefully tailoring a drug carrier to the drug. A tool is needed to help predict the best possible polymer structure for the drug. This is not easy, but an initial estimate can be obtained using the group contribution method to determine approximate partial solubility parameters. In this approach, the polymer and drug are essentially dissected into their different functional groups, which then are used to determine dispersion forces, dipole-dipole interactions, and hydrogen bonding of polymer and drug.[11] This approach frequently has been employed to predict the most suitable polymeric drug carrier,[12–13] but one also needs to exercise caution since many aspects are not taken into account resulting in unsuitable predictions. More refined approaches are based on molecular dynamics simulation,[14] which can reveal the critical role of H-bonding, an interaction that is often more crucial than hydrophobic forces to achieve high drug loading.[15] Further theories, based on a free

energy model, describe the solubilization of low molecular weight compounds in micelles. Based on this information, one can derive conclusions on the distribution of the drug in the micelle. aggregation number, the size of the micelle, the effect on micelle stability, and the maximum extent of solubilization.[16] A summary of various computational approaches can be found in a review article by Allen and co-workers.[17]

Methods: Micelle Drug Loading

Once a suitable polymer has been identified, the drug must be loaded into the micelles.[7,18] Direct mixing of the hydrophobic drug and the micelle in water, although suitable for some selected systems, is rarely capable of dissolving both the drug and polymer. Therefore, other techniques must be used to ensure solubilization of both the drug and the polymer. A common solvent is capable of fully dissolving both the drug and the block copolymer into the unimeric state (single block copolymers). A clear solution can serve as an initial indication the polymer has dissolved, but it is advisable to test for the absence of micelles or other aggregates using light scattering techniques to ensure full solvation. Examples of commons drug-loading techniques are described in **Figure 1**.

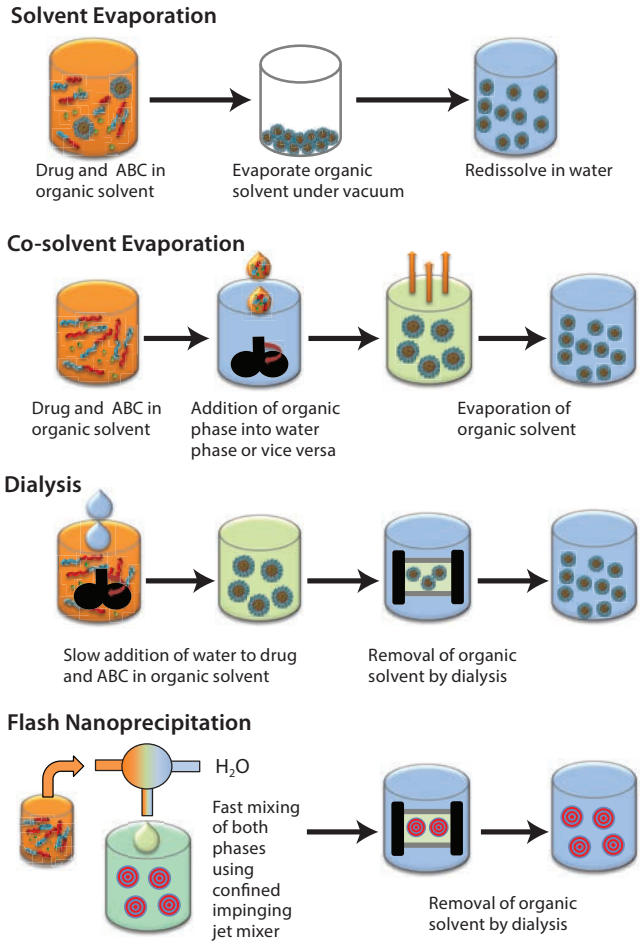

Figure 1. Techniques for drug loading of micelles

Solvent Evaporation

In the solvent evaporation technique, polymer and drug are dissolved in an organic solvent with a low boiling point, followed by evaporation and subsequent dehydration.[19] The chosen organic solvent is selective toward one block, which results in the formation of micelles in non-aqueous solutions. The outcome is usually determined by the type of solvent, the concentration of polymer and drug, and the rate of evaporation. The limitation of this approach lies in the limited choice of solvents, and there is no guarantee the resulting particles will be well-defined core-shell particles that can be easily redissolved in water.

Example Method

1. Dissolve 2 mg of drug and 20 mg of polymer in methanol (or any other low-boiling solvent that can dissolve both components).
2. Evaporate solvent under vacuum.
3. Add distilled water, incubate at 40 °C for 10 min, and vortex to obtain a clear solution.

Co-solvent Evaporation

Co-solvent evaporation proceeds by adding water directly to the organic solvent to cause the self-assembly of the micelle and encapsulation of the drug. The outcome is controlled by the type of solvent, the ratio between organic solvent and water, the concentration of water and drug, rate of solvent evaporation, and the order and rate of mixing.[20] This approach is limited by the choice of solvent, but usually results in higher drug encapsulation efficiencies.

Example Method

1. Dissolve 20 mg of polymer and 2 mg of drug in 1 mL of acetone, THF, or acetonitrile.
2. Add 2 mL of water dropwise to the organic solvent (or vice versa).
3. Mix for 4 h, followed by the evaporation of the organic solvent.

Dialysis

Dialysis is probably the most versatile and most common technique used for drug encapsulation since it allows the use of high-boiling solvents such as DMSO, which is removed by dialysis and replaced with water. Although this approach is applicable to many solvent systems, the drug loading efficiency is usually lower than the co-solvent evaporation and the technique can be time-consuming. The slow process can aid the formation of thermodynamically stable morphologies. A final dialysis step to remove solvent and free drug is often crucial to obtain a product free of organic solvent while maintaining maximum drug loading. However, while extensive dialysis can assist in the thorough purification of the product, it also can cause the release of the already encapsulated drug and result in low drug encapsulation efficiencies.

Example Method

1. Dissolve 20 mg of polymer and 2 mg of drug in 1 mL of DMF.
2. Add 5 mL of water slowly, with the help of a syringe pump, if possible, to control the rate of water addition.
3. Dialyze against water using a tubular cellulose membrane (**Sigma Prod. No.** Z726176).

Flash Nanoprecipitation

Flash nanoprecipitation is a relatively new technique that offers a more rapid solution than other time-consuming methods. Fast mixing and precipitation into a non-solvent for the drug and one polymer block results in a kinetically trapped structure. Although the resulting structures do not have well-defined internal phase boundaries, as would be the case in thermodynamically stable structures, the approach provides an alternative to achieve a fast throughput.[21]

Example Method

1. Prepare a solution of 40 mg of polymer and 20 mg of drug in 1 mL of THF.
2. Use a confined impinging jet mixer to mix the solution with 1 mL of water.
3. Introduce the exit stream into 8 mL of water:THF (9:1 v/v%).
4. Dialyze against water using a tubular cellulose membrane.

Characterization

Independent of the technique, the characterization of drug-loaded micelles is similar to other nanoparticles. Parameters of interest are the drug encapsulation efficiency (EE) and the drug-loading capacity (LC) (see **Equation 2**):

$$EE\% = \frac{W_L}{W_o} \qquad LC\% = \frac{W_L}{W_N} \qquad (2)$$

where W_L is the weight of loaded drug, W_0 the quantity of drug initially added, and W_N the weight of the nanoparticle.

The International Organization for Standardization (ISO) published a catalog of properties for the full characterization of nanoparticles. The *Guidance on Physico-chemical Characterization of Engineered Nanoscale Materials for Toxicologic Assessment* (ISO/TR 13014:2012) includes dimensions, shape, specific surface area, surface charge, composition, and purity, among others. While most of these points apply to micelles as well, the evaluation of the stability of the micelle can be considered a crucial aspect when trying to evaluate micelles for drug delivery purposes. It has been shown the dynamic behavior of micelles can affect their cellular uptake[22] and their rate of exocytosis.[23] The physico-chemical characterization of nanoparticles is then complemented by the *Compilation and Description of Toxicological Screening Methods for Manufactured Nanomaterials* (ISO/TR 16197:2014), which contains a list of recommended experiments to understand toxicity, accumulation, and other factors. The reader is referred to comprehensive publications on this topic, which describe background and also give practical advice.[24,25]

Multicelluar spheroids are an established drug discovery technique that is making its way into the nanomedicine area, including the testing of drug-loaded micelles.[26–28] Interestingly, while some drug-loaded micelles can have poor performance in monolayer cell models, the results in a three-dimensional (3D) cell culture experiment can differ noticeably.[26] The key to this behavior is the differences in penetration of drugs and micelles into the multicellular spheroid. While this aspect only plays a minor role in two-dimensional (2D) models, it becomes one of the main parameters in understanding enhanced delivery in a 3D environment.[29] These 3D models can be further combined with 2D models to create sophisticated systems that mimic the tumor microenvironment to simulate the behavior of micelles *in vivo*.[30]
A typical procedure to test the toxicity of drug-loaded micelles is outlined below. Although the researcher can choose from a range of ways to culture spheroids,[31] only the "hanging drop" method is discussed here. The hanging drop method is a convenient method of producing a family of relatively uniform spheroids without the use of specialized equipment.

Method: Characterization of Toxicity with Spheroids

Typical steps for culturing spheroids in this manner include:

1. Pre-determine the seeding number of cells required for each spheroid (usually 1,000–2,000 cells/spheroid).
2. Prepare a cell suspension with a per mL concentration 100× that of the initial seeding cell number where the suspension is prepared in the growth medium used to culture the cells.
3. Place 10 µL of the well-mixed cell suspension on the inside surface of the lid of a petri dish.
4. Repeat until the required number of seeding droplets are placed.
5. Flip the petri dish lid upside down so that the seeding droplets are "hanging" and place on top of a petri dish filled with 15 mL of sterile PBS.
6. Incubate at 37 °C and 5% CO_2 for a minimum of 3 days without disturbing the petri dish.
7. Check the spheroids daily until the desired size has been reached (usually 300–400 µm in diameter).
8. Once culturing is complete, move the spheroids into a 96-well plate filled with 200 µL of medium/well.
9. Culture the spheroids for one more day at 37 °C and 5% CO_2 with slow rotation of the plate on a 4-way mixer during that time.

Once the spheroids have been cultured and are of the desired shape and size, the micelle formulation can be loaded for testing:

1. Remove 170 µL of medium from each well in the 96-well plate containing the spheroids.
2. Add 100 µL of twice-concentrated culture medium and 100 µL of the twice-concentrated micellar solution to each well.
3. Incubate for the desired amount of time at 37 °C and 5% CO_2.
4. At the desired time points, move the spheroids to be tested into a new plate and wash with PBS.
5. Measure the viability of the spheroids via several methods, including determination of DNA amount or measuring acid phosphatase activity.[32]

Additional tips for culturing spheroids include:

1. Use the surface tension of the droplet to naturally maintain its shape when initially placing it on the petri dish lid.
2. Place droplets at least 1 cm apart to give room for the droplets to spread slightly upon culturing.
3. Prepare at least 25% more spheroids than required in case of undesired spheroid shape or size.

Examples of spheroids are provided in **Figure 2**. Confocal microscopy can be used to visualize micelle penetration if fluorescence is incorporated into the polymeric structure.

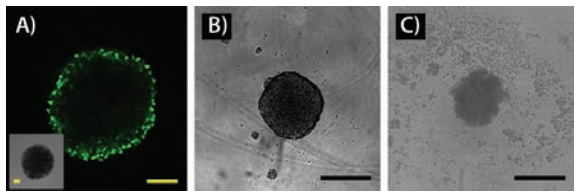

Figure 2. A) Penetration of uncrosslinked and fluorescent micelles into prostate cancer (LNCaP) spheroids as visualized by confocal microscopy at a depth of 90 µm (yellow scale bar = 100 µm); **B**) LNCaP spheroids after 4 days culture (black scale bar = 300 µm); **C**) LNCaP spheroids after 14 days treatment with the uncrosslinked micelles which have been conjugated with paclitaxel.

Conclusions

The delivery of drugs using polymeric micelles has now matured into a well-established field. Compatibility between the drug and polymer is the key to success in obtaining maximum loading efficiency. The scientist can choose from a range of tools to load the drug. Although many drugs can be loaded using the above techniques, it should be noted that a range of drugs—such as drugs that have a low drug-loading efficiency—are best delivered by conjugating them to the block copolymer directly, instead of relying on physical attraction alone. It must be noted that this article has not touched upon the benefits of crosslinking micelles.[2,33] As briefly discussed here, the dynamic properties of the micelles and the potential disassembly may affect the interaction with biological media, and crosslinking may circumvent the issue as it may enhance characteristics such as cellular uptake,[22,23] movement in multicellular tumors,[29] and *in vivo* circulation.[33] In summary, polymeric micelles provide limitless avenues of modification possibilities and represent a versatile method of delivering a wide range of drugs.

References

(1) Xiong, X.-B.; Falamarzian, A.; Garg, S. M.; Lavasanifar, A. *J. Controlled Release* **2011**, *155*, 248–261.
(2) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, *41*, 2545–2561.
(3) Cabral, H.; Kataoka, K. *J. Controlled Release* **2014**, *190*, 465–476.
(4) Lu, Y.; Park, K. *Int. J. Pharm.* **2013**, *453*, 198–214.
(5) Siegwart, D. J.; Oh, J. K.; Matyjaszewski, K. *Prog. Polym. Sci.* **2012**, *37*, 18–37.
(6) Gregory, A.; Stenzel, M. H. *Prog. Polym. Sci.* **2012**, *37*, 38–105.
(7) Kowalczuk, A.; Trzcinska, R.; Trzebicka, B.; Müller, A. H. E.; Dworak, A.; Tsvetanov, C. B. *Prog. Polym. Sci.* **2014**, *39*, 43–86.
(8) Qian, F.; Huang, J.; Hussain, M. A. *J. Pharm. Sci.* **2010**, *99*, 2941–7.
(9) Yokoyama, M.; Fukushima, S.; Uehara, R.; Okamoto, K.; Kataoka*, K.; Sakurai, Y.; Okano, T. *J. Controlled Release* **1998**, *50*, 79–92.
(10) Falamarzian, A.; Lavasanifar, A. *Macromol. Biosci.* **2010**, *10*, 648–656.
(11) J. Liu, Y. X., C. Allen. *J. Pharm. Sci* **2004**, *93*, 132–143.
(12) Kim, Y.; Liemmawa, E. D.; Pourgholami, M. H.; Morris, D. L.; Stenzel, M. H. *Macromolecules* **2012**, *45*, 5451–5462.
(13) Sharma, A.; Soliman, G. M.; Al-Hajaj, N.; Sharma, R.; Maysinger, D.; Kakkar, A. *Biomacromolecules* **2012**, *13*, 239–252.
(14) Patel, S. K.; Lavasanifar, A.; Choi, P. *Biomacromolecules* **2009**, *10*, 2584–2591.
(15) Schulz, A.; Jaksch, S.; Schubel, R.; Wegener, E.; Di, Z.; Han, Y.; Meister, A.; Kressler, J.; Kabanov, A. V.; Luxenhofer, R.; Papadakis, C. M.; Jordan, R. *ACS Nano* **2014**, *8*, 2686–2696.
(16) Nagarajan, R.; Ganesh, K. *Macromolecules* **1989**, *22*, 4312–4325.
(17) Huynh, L.; Neale, C.; Pomès, R.; Allen, C. *Nanomedicine: Nanotechnology, Biology and Medicine* **2012**, *8*, 20–36.
(18) Gaucher, G.; Dufresne, M.-H.; Sant, V. P.; Kang, N.; Maysinger, D.; Leroux, J.-C. *J. Controlled Release* **2005**, *109*, 169–188.
(19) Lavasanifar, A.; Samuel, J.; Kwon, G. S. *J. Controlled Release* **2001**, *77*, 155–160.
(20) Aliabadi, H. M.; Elhasi, S.; Mahmud, A.; Gulamhusein, R.; Mahdipoor, P.; Lavasanifar, A. *Inter. J. Pharm.* **2007**, *329*, 158–165.
(21) York, A. W.; Zablocki, K. R.; Lewis, D. R.; Gu, L.; Uhrich, K. E.; Prud'homme, R. K.; Moghe, P. V. *Adv. Mater.* **2012**, *24*, 733–739.
(22) Kim, Y.; Pourgholami, M. H.; Morris, D. L.; Stenzel, M. H. *Biomacromolecules* **2012**, *13*, 814–825.
(23) Kim, Y.; Pourgholami, M. H.; Morris, D. L.; Lu, H.; Stenzel, M. H. *Biomater Sci* **2013**, *1*, 265–275.
(24) Hall, J. B.; Dobrovolskaia, M. A.; Patri, A. K.; McNeil, S. E. *Nanomedicine (Lond)* **2007**, *2*, 789–803.
(25) McNeil, S. Characterization of Nanoparticles Intended for Drug Delivery. Humana Press: **2011**; Vol. *697*.
(26) Du, A. W.; Lu, H.; Stenzel, M. H. *Biomacromolecules* **2015**, *16*, 1470–1479.
(27) Sarisozen, C.; Abouzeid, A. H.; Torchilin, V. P. *Eur J Pharm Biopharm* **2014**, *88*, 539–50.
(28) Jiang, Y.; Lu, H.; Khine, Y. Y.; Dag, A.; Stenzel, M. H. *Biomacromolecules* **2014**, *15*, 4195–4205.
(29) Lu, H.; Utama, R. H.; Kitiyotsawat, U.; Babiuch, K.; Jiang, Y.; Stenzel, M. H. *Biomaterials Science* **2015**.
(30) Gao, H.; Yang, Z.; Zhang, S.; Pang, Z.; Liu, Q.; Jiang, X. *Acta Biomater* **2014**, *10*, 858–67.
(31) Hickman, J. A.; Graeser, R.; de Hoogt, R.; Vidic, S.; Brito, C.; Gutekunst, M.; van der Kuip, H. *Biotechnol J* **2014**, *9*, 1115–28.
(32) Friedrich, J.; Seidel, C.; Ebner, R.; Kunz-Schughart, L. A. *Nature Protocols* **2009**, *4*, 309–324.
(33) van Nostrum, C. F. *Soft Matter* **2011**, *7*, 3246–3259.

Diblock Copolymers

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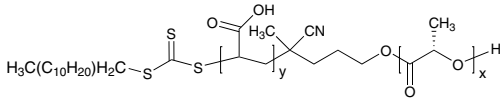
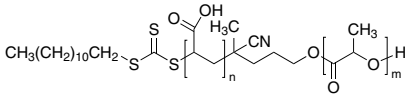
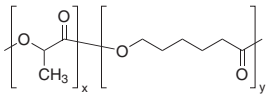
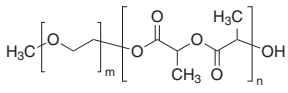
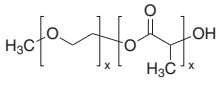
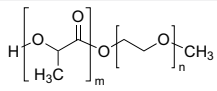
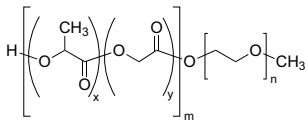
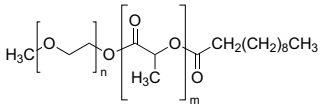
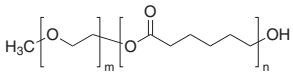
Name	Structure	Molecular Weight/Viscosity	Degradation Time	Prod. No.
Poly(*L*-lactide-block-acrylic acid)		PAA average M_n 18,000 PLA average M_n 4,500	-	805718-1G
		PAA average M_n 18,000 PLA average M_n 10,000	-	799246-250MG
Poly(D,L-lactide-*block*-acrylic acid)		PAA average M_n 18,000 PLA average M_n 5,000	-	798126-1G
Poly(DL-lactide-*co*-caprolactone)		inherent viscosity 0.7-0.9 dL/g in chloroform, DL 86%	-	457639-5G
		inherent viscosity 0.7-0.9 dL/g in chloroform, DL 40%	-	457647-5G
Poly(L-lactide)-block-poly(ethylene glycol)methyl ether		PEG average M_n 5,000 PLA average M_n 5,000	-	570281-250MG 570281-1G
Poly(ethylene glycol)-*block*-polylactide methyl ether		PEG average M_n 350 PLA average M_n 1,000	-	659665-1G
		PEG average M_n 750 PLA average M_n 1,000	-	659657-1G
Poly(ethylene glycol) methyl ether-*block*-poly(D,L lactide)		PEG average M_n 2,000 PLA average M_n 2,200 average M_n 4,000 (total)	2-4 weeks	764779-1G

Name	Structure	Molecular Weight/Viscosity	Degradation Time	Prod. No.
Poly(ethylene glycol) methyl ether-*block*-poly(lactide-*co*-glycolide)		PEG M_n 2,000 PLGA M_n 4,000 average M_n 6,000 (total)	1-4 weeks	764825-1G
		PEG average M_n 5,000 PLGA M_n 10,000 average M_n 15,000 (total)	1-4 weeks	765139-1G
		PEG average M_n 2,000 PLGA average M_n 15,000 average M_n 17,000 (total)	1-4 weeks	764760-1G
		PEG average M_n 5,000 PLGA M_n 55,000 average M_n 60,000 (total)	1-4 weeks	764752-1G
Poly(ethylene glycol) methyl ether-*block*-poly(D,L lactide)-*block*-decane		PEG average M_n 2,000 PLA average M_n 2,000 average M_n 4,000 (total)	2-5 weeks	764736-1G
Poly(ethylene glycol)-*block*-poly(ε–caprolactone) methyl ether		PCL average M_n 5,000 PEG average M_n 5,000 average M_n 10,000 (total)	>12 months	570303-250MG 570303-1G
		PCL average M_n 13,000 PEG average M_n 5,000 average M_n 18,000 (total)	>12 months	570311-250MG 570311-1G
		PCL average M_n 32,000 PEG average M_n 5,000 average M_n 37,000 (total)	>12 months	570338-250MG 570338-1G

Triblock Copolymers

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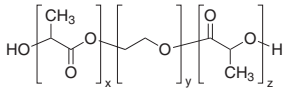
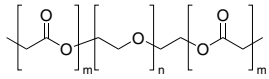
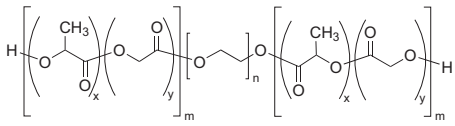
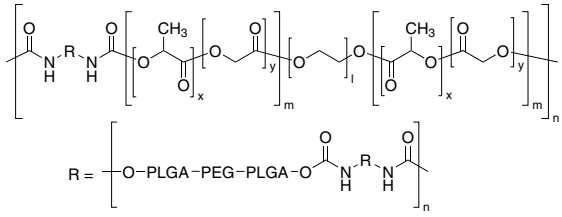
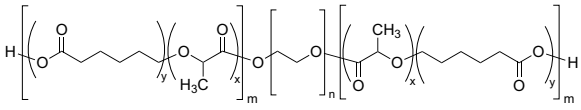
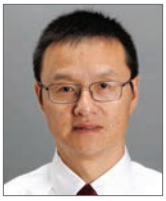
Name	Structure	Molecular Weight/Viscosity	Degradation Time	Prod. No.
Polylactide-*block*-poly(ethylene glycol)-*block*-polylactide		PEG average M_n 900 PLA average M_n 1,500 average M_n 1,500-900-1,500	<12 months	659630-1G
		PEG average M_n 10,000 PLA average M_n 1,000 average M_n 1,000-10,000-1,000	<12 months	659649-1G
Polyglycolide-*block*-poly(ethylene glycol)-*block*-polyglycolide		PEG average M_n 400 PEG:Gly 8:92	-	790230-1G
		PEG average M_n 400 PEG:Gly 12:88	-	790222-1G
Poly(lactide-*co*-glycolide)-*block*-poly(ethylene glycol)-*block*-poly(lactide-*co*-glycolide)		PEG average M_n 1,000 PLGA average M_n 2000 average M_n 1,000-1,000-1,000	1-2 weeks	764787-1G
		PEG average M_n 1,000 PLGA average M_n 2,200 average M_n 1100-1000-1100	2-3 weeks	764817-1G
Poly(*D,L*-lactide-co-glycolide)-block-poly(ethylene glycol)-block-poly(*D,L*-lactide-co-glycolide) based poly(ether ester urethane)		PEG average M_n 400 average M_n 6,000-12,000	<4 months	790257-1G 790257-5G
		PEG average M_n 400 average M_n 6,000-15,000	<4 months	790249-5G 790249-1G
		PEG average M_n 400 average M_n 8,000-20,000	-	790265-1G 790265-5G
Poly(lactide-co-caprolactone)-*block*-poly(ethylene glycol)-*block*-poly(lactide-co-caprolactone)		PEG average M_n 5,000 PLCL average M_n 5,700 average M_n 1,000-10,000-1,000	1-2 months	764833-1G

Controlled Release | Targeted Delivery | Solubility Enhancement

BIODEGRADABLE COLLOIDAL CARRIERS
IN DRUG DELIVERY APPLICATIONS

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Introduction

Colloidal carriers are particles or vesicles of nanometer to micron size that facilitate drug delivery. Common colloidal carrier systems include liposomes, polymeric microspheres and nanoparticles, nanocrystals, and microemulsions. Colloidal carriers can be used to improve the therapeutic index of APIs by transporting loaded drugs to the target site and modifying their distribution within the body. Furthermore, colloidal carriers can alter the pharmacokinetics of drug molecules, increase efficacy, reduce toxicity, and provide controlled and sustained release.

Polymeric Microspheres

Polymeric microsphere drug carriers are spherical particles in the size range of several to hundreds of microns that can protect unstable drugs pre- and post-administration. Microspheres have the ability to release a drug continuously over time,[1] thereby providing a prolonged therapeutic effect and reducing the dosing frequency. In addition to controlled release, microspheres allow for the targeted drug delivery of potent drugs at reduced concentrations, thereby minimizing systemic exposure and adverse side effects. Finally, polymeric microspheres facilitate manipulation of *in vivo* behavior, pharmacokinetic profile, tissue distribution, and cellular interaction of the drug.[2]

Microspheres are typically comprised of biodegradable polymers such as poly(lactide-co-glycolide) (PLGA), polylactic acid (PAA), polylactide (PLA), and polycaprolactone (PCL). These polymers degrade *in vivo* by hydrolysis of their ester backbone into non-toxic products, which are excreted by the kidneys or eliminated as CO_2 and water through biochemical pathways. PLGA microspheres have been widely used to encapsulate drug molecules and have been used as long-acting, sustained-release pharmaceutical formulations. There are several drug-loaded PLGA microspheres approved by the FDA and marketed for clinical use. For example, depot products, such as luprolide acetate microspheres used for the treatment of prostate cancer and endometriosis, can be subcutaneously administered at 1-month, 3-month, or 6-month intervals. When drug-loaded PLGA microspheres are administered, the PLGA polymer starts to degrade *in vivo*, and as it degrades the drug molecules are gradually released from the microspheres. The drug release rate can be modulated by the selection of the type of PLGA polymer and by adjusting the encapsulation process. For example, the following parameters can affect the drug release profile:

- The ratio of lactide to glycolide (L/G ratio) in the PLGA polymers; e.g., PLGA with an L/G ratio of 50:50 have the fastest drug release.
- The molecular weight or inherent viscosity of the PLGA polymer, where higher molecular weight provides slower drug release.
- The terminal group of the PLGA polymer, where carboxyl-terminated PLGA polymers offer faster drug release compared to ester-terminated PLGA.

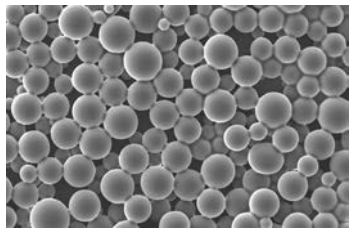

Figure 1. Image of API-loaded PLGA microspheres.

Polymeric Nanoparticles

Polymeric nanoparticles (NP), either plain or drug loaded, are typically less than 1 micron in size. The use of API-loaded polymeric nanoparticles for intravenous administration is a promising approach for achieving the controlled release and site-specific delivery of drugs. The nanoparticle delivery system can be designed to maintain appropriate therapeutic concentration in the bloodstream (controlled release) or to target a specific cell type (e.g., bone marrow, blood cells). Various types of APIs, including small molecule drugs and biologic compounds, can be incorporated into PLGA polymer nanoparticles by either microencapsulation or surface conjugation. Nanoencapsulation can protect the API from early degradation, facilitate cell entry, and increase solubility and bioavailability.

The surface properties of intravenously injected particles are important factors determining *in vivo* organ distribution and fate. Furthermore, surface modification can be an effective approach to targeting specific

tissues. Surface modification of nanoparticles with polyethylene glycol (PEG) can be used to prolong the *in vivo* circulation lifetime of drug-loaded nanoparticles. PEGylation of the nanoparticle can be accomplished by adding a copolymer containing PEG chains during the nanoparticle fabrication process. For example, the addition of an ethylene glycol monomer during lactide and glycolide copolymerization can lead to a PEGylated PLGA polymer. PEGylation can increase nanoparticle hydrophilicity and improve degradation rate and crystallization.[3] In addition to being biocompatible, PEG is resistant to immunological recognition. PEG units on the NP surface prevent opsonin-NP binding, thus preventing the nanoparticles from being recognized by monocytes and macrophages and, therefore, increasing circulation time in the body.[4] In some circumstances nanoparticles have been shown to remain in circulation 40× longer when coated with PEG compared to uncoated nanoparticles.[5] Other advantages of PEGylated nanoparticles include increased drug loading of hydrophilic drugs, reduced initial burst and improved bioavailability.[6] PEGylated nanoparticles have been used as carriers for vaccine and protein APIs and are particularly useful in both sustained/controlled release and targeted drug delivery systems. Currently, there are more than 35 U.S. FDA-approved products utilizing PEG in their biomedical applications.[4]

Nanoparticles can also facilitate the crossing of the blood brain barrier (BBB). Surfactants such as Polysorbate 80 and Poloxamer 188 have been shown to facilitate the BBB crossing of drug molecules encapsulated in polybutyl cyanoacrylate nanoparticles or solid lipid nanoparticles.[7]

In some nanoparticle formulations, the API is attached to the surface instead of being encapsulated inside the particle. For example, a peptide for ocular delivery (POD) and a human immunodeficiency virus transactivator were conjugated to the surface of PLGA nanoparticles, and the conjugate was found to improve ocular drug bioavailability.[8] The conjugation can be done by reacting the terminal functional group (e.g., terminal COOH) on the PLGA molecule with a reactive group (e.g., amino) on the peptide, API or protein. Finally, in some cases, PLGA nanoparticles themselves can have therapeutic effects against certain diseases.[2]

Microgels and Nanogels

Hydrogel particles, including microgels and nanogels, consist of crosslinked networks of hydrophilic polymer chains that form colloidal gels. Hydrogel particles are made from natural or synthetic polymeric networks, are highly absorbent, and can contain over 90% water. Crosslinking between polymer chains is either chemically or physically induced and prevents the dissolution of these networks in water.[11] The release of the loaded API from the hydrogel particles may occur through diffusion, hydrogel matrix swelling, or chemical reactivity of the drug/matrix. The physical properties of microgels and nanogels (i.e., swelling, permeation, mechanical strength, and surface characteristics) can be optimized by structural modification.[11] Many hydrogel particles are also environmentally sensitive and have the ability to respond to changes of pH, temperature, or the concentration of metabolites, and release their load as a response to these stimuli for controlled drug release.

In drug delivery, microgels and nanogels fill a unique niche because they can encapsulate water-soluble, small molecule APIs that are difficult to encapsulate using traditional biodegradable polymeric particles comprised of PLGA and PCL. As a result, these gels are particularly suitable for the sustained release of water-soluble drugs or proteins. Other advantages of hydrogel particles include their high drug loading and activity, biocompatibility, and biodegradability. Additionally, the manufacturing of colloidal gels does not require organic solvents, eliminating toxicity risks and the potential for protein denaturation.

Natural polymers such as chitosan and alginate have been studied extensively for the preparation of hydrogel nanoparticles. Hydrogel nanoparticles based on synthetic polymers including poly (vinyl alcohol) (PVA), PEG, poly (ethyleneimine) (PEI), poly vinyl pyrrolidone, and poly-N-isopropylacrylamide have also been used for drug delivery.

Hydrogel systems have various applications including oral, transdermal, nasal, rectal, and ocular drug delivery. Hydrogel membranes facilitate transdermal drug delivery through the skin at a predetermined and controlled rate. They are advantageous as they prevent the first pass metabolism effect.[12] Additionally, hydrogel delivery systems can increase the bioavailability of ophthalmic drugs by increasing the contact time of the drugs with cornea.[13]

Embolization therapy is another application of hydrogel microspheres. It is typically used to prevent the growth of solid tumors by blocking the blood supply to the feeding artery. For example, trans-arterial chemical embolization is an application where anticancer drug loaded particles are injected into the feeding artery of cancer tumors. In addition to blocking the blood supply to the tumor, they release the anticancer drugs at high concentrations inside the tumor.[14]

Methods: Colloidal Carrier Fabrication

Colloidal carriers can be fabricated using a variety of techniques. The method for synthesis should be selected based on the type and nature of the drug to be encapsulated as well as the desired particle size, delivery route, and release characteristics for the final formulation.

Nanoprecipitation

Nanoprecipitation is a facile and low energy process for the preparation of polymeric nanoparticles. It is based on interfacial deposition due to the displacement of a solvent with the non-solvent. Miscibility of the solvents and the dilute polymer solutions are required for nanopreciptation.[13] For drug delivery applications, nanoprecipitation is often used for small-scale preparation of nanoparticles of polylactic acid, PLGA, and polycaprolactone. It is well-suited for hydrophobic APIs.

In a typical nanoprecipitation process, a polymer (e.g., PLGA) (and hydrophobic drug, if desired) is dissolved in a solvent that is miscible with water (e.g., acetone). The polymer/drug solution is added dropwise to an aqueous solution under continuous stirring. Surfactants or polymer stabilizers may be added to the aqueous solution to stabilize the nanoparticles during formation. Common surfactants and stabilizers include TWEEN® 20, sodium dodecyl sulfate (SDS), PVA, hydroxymethylcellulose (HPMC), and Pluronic® F-68. The organic solvent is removed by evaporation or repetitive washing. Additional washing steps may be performed to remove surfactant and unincorporated API. The purified nanoparticles can be lyophilized for storage.

The following is a typical protocol for preparing PLGA nanoparticles, of approximately 200 nm, using a one-step nanoprecipitation.

1. PLGA polymer with an L/G ratio of 50/50, COOH terminated, and inherent viscosity of 0.55–0.75 dL/g (additional PLGA polymers may be substituted, such as **Aldrich Prod. No.** 719900) is dissolved in acetone (**Aldrich Prod. No.** 179124).
2. Prepare a polyvinyl alcohol (PVA) solution by dissolving the appropriate amount of PVA (M_w 85,000–124,000, 87–89% hydrolyzed, **Aldrich Prod. No.** 363081) in DI water. A typical concentration is 1%.
3. Transfer 100 mL of the 1% PVA solution prepared in Step 2 to a 500 mL beaker equipped with a magnetic stir bar. Stir the PVA solution at 400 rpm.

4. Using a disposable pipette, slowly and dropwise add 10 mL of the PLGA solution into the stirring PVA solution. The nanoparticles form on contact when the PLGA solution is added.
5. After the PLGA solution is completely added to the PVA solution, continue stirring in a fume hood for 3 h to allow acetone evaporation.
6. Wash the nanoparticles three times using refrigerated centrifugation and follow with lyophilization.
7. Store lyophilized PLGA nanoparticles dry at –20 °C.

Emulsification Process

Emulsification can be used to prepare plain or drug-loaded polymeric microspheres and nanoparticles. Depending on the type of drug to be loaded, either a single emulsion or double emulsion may be used.

Single Emulsion

In a single emulsion, the polymer (e.g., PLGA, PCL) is dissolved in a solvent that is not miscible with water. If a drug is to be encapsulated, it is preferably hydrophobic and solvent soluble. The hydrophobic drug is dissolved in the same solution as the polymer. The polymer/drug solution is emulsified in an aqueous solution containing a surfactant or a polymeric stabilizer (as noted earlier). Emulsification can be completed by sonication, magnetic or mechanical stirrer, rotor stator, high-pressure homogenizer, or microfluidizer. After the oil-in-water emulsion is formed, the solvent is removed by evaporation or extraction. The particles can be washed to remove the surfactant and possible unincorporated drug molecules, and then lyophilized.

The single emulsion process can also be used to prepare nanocrystals of poorly soluble compounds. For example, a single emulsion process was used to prepare albumin bound paclitaxel nanoparticles.[14–16]

The following is a typical protocol for preparing PLGA nanoparticles using a single-emulsion process:

1. Prepare a 5% PLGA solution by dissolving PLGA polymer with an L/G ratio of 50/50, COOH terminated, and inherent viscosity of 0.55–0.75 dL/g (additional PLGA polymers may be substituted, such as **Aldrich Prod. No.** 719900) in methylene chloride (**Sigma Prod. No.** 443484).
2. Prepare a 1% solution of polyvinyl alcohol (PVA) (M_w 85,000–124,000, 87–89% hydrolyzed, **Aldrich Prod. No.** 363081) solution in DI water.
3. Mix 5 mL of the 5% PLGA solution prepared in Step 1 with 100 mL of the 1% PVA solution prepared in Step 2 in a 500 mL beaker.
4. Homogenize the mixture of the PLGA and PVA solutions by using an IKA Ultra Turrax High Speed Homogenizer at 18,000 rpm for 2 min.
5. Stir the resulting emulsion at 400 rpm on a stir plate in a fume hood for 3 h to allow the methylene chloride to evaporate.
6. Wash the nanoparticles three times using refrigerated centrifugation and lyophilize.
7. Store lyophilized PLGA nanoparticles dry at –20 °C.

Double Emulsion

For hydrophilic API encapsulation, a double-emulsion technique is necessary to prepare the polymeric microspheres and nanoparticles. The double emulsion is predominately a water-in-oil-in-water emulsion, although in some cases it can be a reverse double emulsion, or an oil-in-water-in-oil. In a typical double-emulsion process, the hydrophilic API is dissolved in an aqueous media and emulsified in the polymer solution to form the first emulsion. The first emulsion is again emulsified in an aqueous solution containing appropriate surfactants or polymer stabilizers to form the second emulsion, or double emulsion. The solvent is then removed by evaporation or extraction processes. The equipment used in single-emulsion processes can be used to generate double emulsions.

In double emulsions, achieving high drug loading of hydrophilic APIs can be challenging since the drug partitions away from the hydrophobic polymer solution into the aqueous surfactant solution. Macromolecular drugs such as proteins and antibodies have all been encapsulated into polymeric microspheres and nanoparticles using a double-emulsion processes.

However, protein aggregation or denaturing may occur during formation. During the microencapsulation process, proteins are constantly exposed to cavitation, heat, solvents, and high shear force, which could lead to aggregation and denaturation.[17,18–20] Alternative techniques have been pursued to encapsulate protein drugs into microspheres and nanoparticles and are reviewed elsewhere.[19–29]

The following is a typical protocol for preparing API-loaded PLGA nanoparticles using a double-emulsion process:

1. Prepare a 1% bovine serum albumin (BSA) solution by dissolving BSA (lyophilized powder, **Sigma Prod. No.** 05470) in DI water.
2. Prepare a 5% PLGA solution by dissolving PLGA polymer with an L/G ratio of 50/50, COOH terminated, and inherent viscosity of 0.55–0.75 dL/g (additional PLGA polymers may be substituted, such as **Aldrich Prod. No.** 719900) in methylene chloride. Prepare a 1% PVA (M_w 85,000–124,000, 87–89% hydrolyzed, **Aldrich Prod. No.** 363081) solution in DI water.
3. Mix 0.5 mL of the BSA solution prepared in Step 1 and 5 mL of the 5% PLGA solution prepared in Step 3 in a 15 mL glass vial.
4. Homogenize the PLGA and BSA solution using an IKA Ultra Turrax High Speed Homogenizer at 20,000 RPM for 25 seconds.
5. Mix the resulting emulsion with 100 mL of the 1% PVA solution prepared in Step 3 in a 500 mL beaker.
6. Homogenize the mixture of the first emulsion and the PVA solution using an IKA Ultra Turrax High Speed Homogenizer generator at 8,000 rpm for 2 min.
7. Stir the resulting double emulsion on a stir plate at 400 rpm in a fume hood for 3 h to allow the methylene chloride to evaporate.
8. Wash the nanoparticles three times using refrigerated centrifugation. Follow with nanoparticle lyophilization.
9. Store lyophilized BSA-PLGA nanoparticles dry at –20 °C.

Spray Drying

In spray drying, the feed (a solution, emulsion, or suspension containing the API and the matrix material) is atomized into hot nitrogen, leading to rapid drying and particle formation. The particles are then separated in a cyclone and/or filter bag.

Spray drying has been used extensively by the pharmaceutical industry to formulate solid dispersions to overcome solubility limitations. Crystalline APIs can be encapsulated within polymer microspheres. For example, enteric polymers can be used to protect a drug from harsh gastric conditions or for enhanced delivery to the site of maximum absorption. Similar approaches can be used for taste masking and protecting the drug from physical environments such as light or moisture. These drug-loaded particles are normally in the micron to millimeter range, although the spray-drying process is capable of producing sub-micron or nanometer sized particles.

In addition to enhancing the oral bioavailability of poorly water-soluble compounds, spray drying offers the ability to control particle size, morphology, and other properties with direct effect in Fine Particle Fraction (FPF) and lung deposition. It also allows for a reproducible, controllable, and scalable manufacturing process. Spray drying is the method of choice where abrasion or shearing needs to be avoided, as in the case of biologic compounds. Inhaled insulin formulations of Affrezza (MannKind) and Exubera (Pfizer) are manufactured by spray drying.

The following is a typical spray-drying protocol for the preparation of chitosan microspheres. This method uses a Mini Spray Dryer B-290 (BUCHI Corporation, New Castle, DE, USA) with a 0.7 mm standard nozzle.

1. Dissolve an appropriate amount of chitosan (medium M_w, **Aldrich Prod. No.** 448877) in 1.0% v/v acetic acid (**Aldrich Prod. No.** 695092) solution to prepare a 2.5% (w/v) chitosan solution in a 500 mL Erlenmeyer flask.
2. Use the suction mode as the method of operation. Set the flow rate of the compressed air at 600 L/h, the inlet temperature at 160 °C, and sample flow rate at 700 mL/h.
3. Start the operation. The chitosan solution is fed to the spray dryer, atomized by the force of the compressed air, and blown by heated air to the drying chamber.
4. Collect the dried microspheres. The mean residence time is 1–1.5 seconds.

References

(1) Sahil, K.; Akanksha, M.; Premjeet, S.; Bilandi, A.; Kapoor, B. *Int. J. Res.Pharm. Chem.* **2011**, *1*, 1184.
(2) Ramteke, K.; Jadhav, V. B.; Dhole, S. N. *IOSR J. of Pharm.* **2012**, *2*, 44.
(3) Xiao, R. Z.; Zeng, Z. W.; Zhou, G. L.; Wang, J. J.; Li, F. Z.; Wang, A. M. *Int J Nanomedicine* **2010**, *5*, 1057.
(4) Moffatt, S. *MOJ Proteomics Bioinform* **2015**, *2*, 00037.
(5) van Vlerken, L. E.; Vyas, T. K.; Amiji, M. M. *Pharm Res* **2007**, *24*, 1405–1414.
(6) Xiao, R. Z.; Zeng, Z. W.; Zhou, G. L.; Wang, J. J.; Li, F. Z.; Wang, A. M. *Int J Nanomedicine* **2010**, *5*, 1057.
(7) Sharma, H. S. Progress in Brain Research in *Nanoneuroscience and Nanoneuropharmacology*, **2009**, page 198 and reference therein.
(8) Vasconcelos, A.; Vega, E.; Pérez, Y.; Gómara, M. J.; García, M. L.; Haro, I. *Int. J. Nanomed.* **2015**, *10*, 609.
(9) Ahmed, E. M. *J Adv. Res.* **2015**, *7*, 105.
(10) Giri, T. K.; Thakur, A.; Alexander, A.; Ajazuddin, H.; Badwaik, H.; Tripathi, D. K.; *Acta Pharmaceutica Sinica B* **2012**, *2*, 439.
(11) Genta, I.; Conti, B.; Perugini, P.; Pavanetto, F.; Spadaro, A.; Puglisi, G. *J. Pharm. Pharmacol.* **1997**, *49*, 737–742.
(12) Saralidze, K.; Koole, L. H.; Knetsch, M. L. W. *Materials* **2010**, *3* (6), 3537–3564.
(13) Hornig, S.; Heinze, T.; Becer, C. R.; Schubert, U. S. *J. Mater.Chem.* **2009**, 3838–3840.
(14) Green, M. R.; Manikhas, G. M.; Orlov, S.; Afanasyev, B.; Makhson, A. M.; Bhar, P.; Hawkins, M. J. *Annals of Oncology* **2006**, *17* (8), 1263–1268.
(15) Miele, E.; Spinelli, G. P.; Miele, E.; Tomao, F.; Tomao, S. *Int. J Nanomed* **2009**, *4*, 99–105.
(16) Stinchcombe, T. E. *Nanomedicine* **2007**, *2* (4), 415–423.
(17) Cleland, J.; Jones, A. *Pharm Res* **1996**, *13*, 1464–1475.
(18) Morlocka, M.; Kollb, H.; Winterb, G.; Kissel, T. *Eur J Pharm Biopharm* **1997**, *43*, 29–36.
(19) Pérez, C.; Castellanos, I. J.; Costantino, H. R.; Al-Azzam, W.; Griebenow. K. *J Pharm Pharmacol* **2002**, *54*, 301–303.
(20) Boury, F.; Ivanova, T.; Panaieotov, I.; Proust, J. E.; Bois, A.; Richou, J. *Langmuir* **1995**, *11*, 1636–1644.
(21) Carrasquilloa, K. G.; Stanleya, A. M.; Aponte-Carroa, J. C.; Jésusa, P. D.; Costantinob, H. R.; Bosquesc, C. J.; Griebenow, K. *J. Controlled Release* **2001** *76*, 199–208.
(22) Sáncheza, A.; Villamayora, B.; Guob, Y.; McIverb, J.; Alonso, M. J. *Int J Pharm* **1999**, *185*, 255–266.
(23) Sturesson, C.; Carlfors, J. *J. Controlled Release* **2000**, *67*, 171–178.
(24) Sah, H. *J. Controlled Release* **1999**, *58*, 143–151.
(25) Péan, J-M.; Boury, F.; Venier-Julienne, M-C.; Menei, P.; Proust, J-E.; Benoit, J-P. *Pharm Res* **1999**, *16*, 1294–1299.
(26) Benoita, J-P.; Faisanta, N.; Venier-Juliennea, M-C.; Menei, P. *J. Controlled Release* **2000**, *65*, 285–296.
(27) Jain, R. A. *Biomaterials* **2000**, *21*, 2475–2490.
(28) Iwata, M.; McGinity, J. W. *J Microencapsul* **1992**, *9*, 201–214.
(29) O'donnell, P. B.; Iwata, M.; McGinity, J. W. *J Microencapsul* **1995**, *12*, 155–163.

Poly(lactide-co-glycolide) Copolymers

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Name	Feed Ratio	End Group	Molecular Weight	Degradation Time	Prod. No.
Resomer® RG 502 H, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 50:50	acid terminated	M_w 7,000-17,000	<3 months	719897-1G 719897-5G
Resomer® RG 503 H, Poly(D,L-lactide-co-glycolide)	lactide:glycolide 50:50	acid terminated	M_w 24,000-38,000	<3 months	719870-1G 719870-5G
Resomer® RG 504 H, Poly(*D,L*-lactide-co-gylcolide)	lactide:glycolide 50:50	acid terminated	M_w 38,000-54,000	<3 months	719900-1G 719900-5G
Resomer® RG 502, Poly(*D,L*-Lactide-Co-Glycolide)	lactide:glycolide 50:50	ester terminated	M_w 7,000-17,000	<3 months	719889-1G 719889-5G
Resomer® RG 503, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 50:50	ester terminated	M_w 24,000-38,000	<3 months	739952-1G 739952-5G
Resomer® RG 504, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 50:50	ester terminated	M_w 38,000-54,000	<3 months	739944-1G 739944-5G
Resomer® RG 505, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 50:50	ester terminated	M_w 54,000-69,000	<3 months	739960-1G 739960-5G
Resomer® RG 653 H, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 65:35	acid terminated	M_w 24,000-38,000	<5 months	719862-1G 719862-5G
Resomer® RG 752 H, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 75:25	acid terminated	M_w 4,000-15,000	<6 months	719919-1G 719919-5G
Resomer® RG 756 S, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 75:25	ester terminated	M_w 76,000-115,000	<6 months	719927-1G 719927-5G
Poly(D,L-lactide-co-glycolide)	lactide:glycolide 85:15	ester terminated	M_w 50,000-75,000	<6 months	430471-1G 430471-5G
Resomer® RG 858 S, Poly(*D,L*-lactide-co-glycolide)	lactide:glycolide 85:15	ester terminated	M_w 190,000-240,000	<9 months	739979-1G 739979-5G

Low PDI Polylactides

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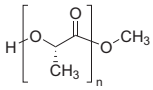
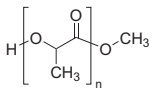
Name	Structure	Molecular Weight	PDI	Degradation Time	Prod. No.
Poly(L-lactide)		average M_n 5,000	≤ 1.2 PDI	>3 years	764590-5G
		average M_n 10,000	≤ 1.1 PDI	>3 years	765112-5G
		average M_n 20,000	≤ 1.1 PDI	>3 years	764698-5G
Poly(D,L-lactide)		average M_n 5,000	≤ 1.1 PDI	<6 months	764612-5G
		average M_n 10,000	≤ 1.1 PDI	<6 months	764620-5G
		average M_n 20,000	≤ 1.3 PDI	<6 months	767344-5G

End-functionalized Low PDI Poly(L-lactide)s

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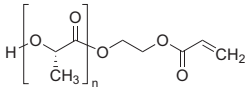
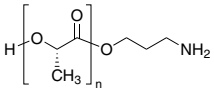
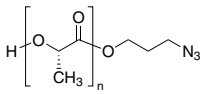
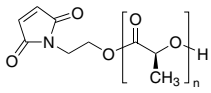
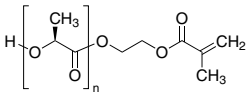
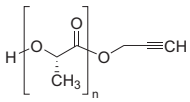
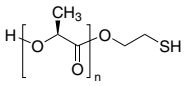
Name	Structure	Molecular Weight	PDI	Prod. No.
Poly(L-lactide), acrylate terminated		average M_n 2,500	≤ 1.2 PDI	775991-1G
		average M_n 5,500	≤ 1.2 PDI	775983-1G
Poly(L-lactide), amine terminated		average M_n 2,500	≤ 1.3 PDI	776378-1G 776378-5G
		average M_n 4,000	≤ 1.2 PDI	776386-1G 776386-5G
Poly(L-lactide), azide terminated		average M_n 5,000	< 1.2 PDI	774146-1G
Poly(L-lactide) N-2-hydroxyethylmaleimide terminated		average M_n 5000	< 1.2 PDI	746517-1G 746517-5G
		average M_n 2,000	≤ 1.2 PDI	746797-1G 746797-5G
Poly(L-lactide) 2-hydroxyethyl, methacrylate terminated		average M_n 2,000	≤ 1.1 PDI	771473-1G 771473-5G
		average M_n 5,500	≤ 1.2 PDI	766577-1G 766577-5G
Poly(L-lactide), propargyl terminated		average M_n 2,000	≤ 1.1 PDI	774162-1G
		average M_n 5,000	≤ 1.1 PDI	774154-1G
Poly(L-lactide), thiol terminated		average M_n 2,500	≤ 1.2 PDI	747386-1G 747386-5G
		average M_n 5,000	≤ 1.2 PDI	747394-1G 747394-5G

Polylactide Block Copoymers

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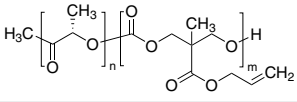
Name	Structure	Molecular Weight	Prod. No.
Poly(L-lactide-*co*-5-methyl-5-allyloxycarbonyl-1,3-dioxan- 2-one)		average M_n 5,000	795259-1G
		average M_n 10,000	795267-1G
		average M_n 40,000	792039-1G

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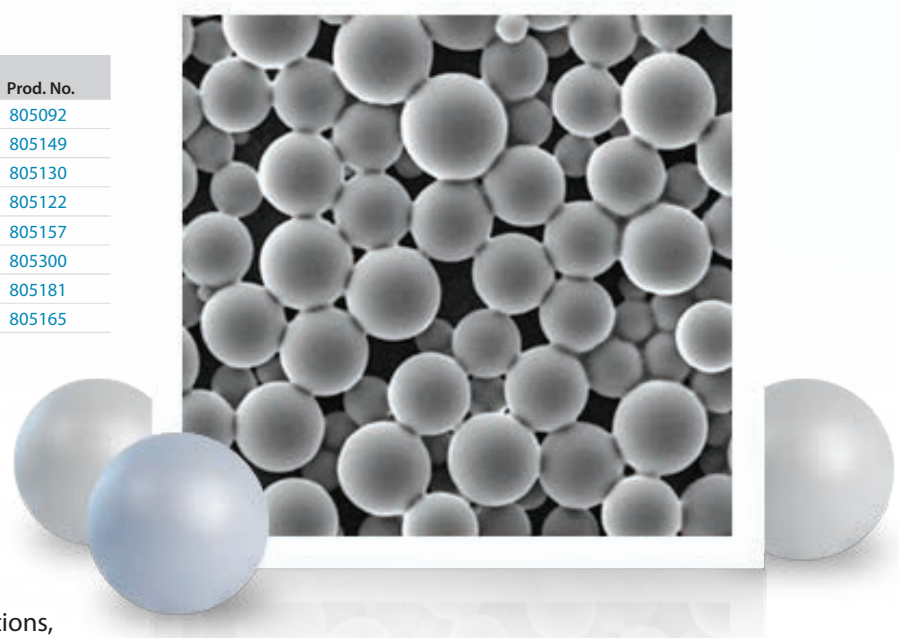
	500 nm	805149
PLGA microspheres	2 µm	805130
	50 µm	805122
Green fluorescent PLGA nanoparticles	100 nm	805157
	500 nm	805300
Green fluorescent PLGA microspheres	2 µm	805181
	50 µm	805165



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LIPID-POLYMER HYBRID NANOPARTICLES
FOR DRUG DELIVERY APPLICATIONS


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Introduction

Since the last decade, there has been a burgeoning interest in the use of nanoparticle-based platforms for drug delivery applications. Nanoparticle-based delivery offers a number of advantages over traditional drug delivery platforms, including the ability to load multiple drugs, attach targeting ligands, enhance drug circulation time, and reduce non-specific drug toxicity. Nanoparticle formulations such as polymeric nanoparticles, liposomes, dendrimers, gold nanoparticles, carbon nanotubes and quantum dots have been widely researched, but only a handful of them have ever reached clinical use.[1]

The inherent advantages of liposomes and polymeric nanoparticles make them the most commonly studied among available drug delivery platforms. For example, liposomes offer excellent biocompatibility,[2] while polymeric nanoparticles possess excellent stability and drug loading capacity.[3] Although the majority of polymeric nanoparticles are still years away from clinical application, researchers have sought to combine the advantages of the two platforms—biocompatibility and high drug loading—by designing hybrids, known as lipid-polymer hybrid nanoparticles (LPNs).[4]

A typical LPN has a core-shell structure, consisting of a polymeric core for loading the cargo, such as small molecule drugs and/or diagnostic molecules, surrounded by a lipid shell for enhanced biocompatibility. The most widely used polymer in the core is poly(lactic-co-glycolic acid) (PLGA) due to its biocompatibility, biodegradability and general drug loading versatility.[5,6] Several lipids, including phosphatidylcholine (PC); 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC); 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE); cholesterol; myristic acid; stearic acid; and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) have been used in the shell, in addition to poly(ethylene glycol) (PEG) lipid conjugates.[5,7]

One-step Synthesis of LPNs

Previously, researchers used various two-step synthesis methods (**Figure 1**), that require the lipid vesicles and polymeric nanoparticle to be separately synthesized before being fused together.[8] This approach gives rise to LPNs with bilayer or multilayer lipid shells. Techniques used to fuse the liposomal shell and the polymeric nanoparticle core together include extrusion, sonication, direct hydration, vortexing, and high-pressure homogenization.

As first demonstrated by Zhang et al., a more convenient one-step synthesis method uses nanoprecipitation and the spontaneous self-assembly of lipid and polymer components (**Figure 1A**), yielding LPNs coated with a lipid monolayer shell.[9] In this method, the polymer and cargo are dissolved in the organic phase (water-miscible organic solvent) and the lipids are dissolved in the aqueous phase. The organic phase is added dropwise to the aqueous phase under continuous stirring, followed by self-assembly at room temperature. To the best of our knowledge, this is the simplest method of synthesizing LPNs currently available.

Alternatively, LPNs can be synthesized using an emulsification technique where the polymer is dissolved in the organic phase (water-immiscible organic solvent) and the lipids are dissolved in the aqueous phase. The solutions are mixed and sonicated to disperse the polymer into droplets and coat the polymers with a layer of lipid. The organic solvent is slowly evaporated under gentle stirring, and the LPNs are then purified for further use.

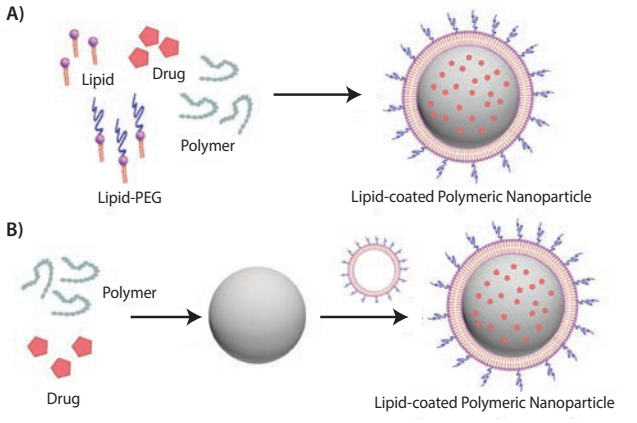

Figure 1. Schematic showing one- and two-step LPN synthesis. **A**) One-step synthesis method. **B**) Two-step synthesis method.

The basic LPN formulation can be modified using targeting moieties to enable site-specific cargo delivery. In many cases, the lipid shell is functionalized using simple conjugation chemistry such as EDC-NHS or thio-maleimide chemistry. For example, LPNs developed by Aravind et al. include AS1411 anti-nucleolin aptamers conjugated to the lipid shell to specifically target cancer cells over-expressing nucleolin receptors.[10] In another example, Clawson et al. developed stimuli-responsive LPNs using a pH-sensitive, lipid-succinate-mPEG coating.[11] In a low-pH tumor microenvironment, disassembly of the PEG layer is triggered causing the internalization of LPNs by cell membrane fusion.

While it is assumed the polymeric core can hold a variety of cargo (sometimes more than one type at once), the lipid shell can also be used to load cargo. Sengupta et al. loaded an anti-angiogenic agent combrestatin-A4 in a lipid shell containing DSPE-PEG, phosphatidylcholine, and cholesterol. At the tumor site, combrestatin-A4 is first released, which shuts down the tumor vasculature. Doxorubicin is later released from the polymeric core to cause cancer cell cytotoxicity.[12]

Summary

LPNs have been used to deliver drugs such as docetaxel,[13] paclitaxel,[14] curcumin,[15] and doxorubicin,[16] in addition to diagnostic molecules for various disease indications. These nanoparticles have characteristics that make them ideal candidates for drug delivery applications, including the ability to load multiple drugs, precisely control drug loading and drug release, and functionalize with targeting moieties.

Although LPNs have been previously formulated using two-step synthesis methods, here we describe a one-step synthesis method that is convenient and reproducible. This method gives rise to LPNs that are less polydisperse in size and whose physicochemical properties can be precisely controlled.[17]

Method: One-step Synthesis of LPNs

The following procedure describes a one-step synthesis method as performed by Prof. Juliana Chan's research group at Nanyang Technological University.

LPNs are synthesized from soybean lecithin, DSPE-PEG, and PLGA using a one-step nanoprecipitation method combined with self-assembly.

1. The aqueous solution is prepared by adding the following to a 4% ethanol aqueous solution in a glass vial (ethanol, **Sigma-Aldrich Prod. No.** E7023):
   - Soybean lecithin consisting of 90–95% phosphatidylcholine (MP Biomedicals, Solon, OH)
   - DSPE–PEG2000–COOH (1,2-distearoyl-*sn*-glycero-3-phospho ethanolamine-N-carboxy(polyethylene glycol)2000) (Avanti, Alabaster, AL).

   The soybean lecithin/DSPE–PEG molar ratio can range from 7:3 to 8.5/1.5.
2. The organic solution is prepared by adding the following to a water-miscible organic solvent such as acetonitrile:
   - Poly(D,L-lactide-co-glycolide) (PLGA) with a 50:50 monomer ratio, ester-terminated, and viscosity of 0.72–0.92 dl/g (Durect Corporation, Pelham, AL). Varying the monomer ratio and viscosity will give rise to LPNs with different sizes, biodegradation rates, and drug-loading and release properties.
   - Small molecule drug such as docetaxel.

   The initial drug weight must not exceed 10–30% of the polymer weight for the drugs to be properly encapsulated by the polymer. The lipid/polymer weight ratio can range from 15%–20%.
3. The aqueous solution is heated to 65 °C on a hotplate stirrer under gentle stirring conditions for 3–5 min.
4. Once the reaction temperature is reached, the organic solution is added dropwise to the aqueous solution under gentle stirring conditions, followed immediately by vigorous vortexing for 3 min.
5. The mixture is returned to gentle stirring conditions and the LPNs are allowed to self-assemble for 2 h at room temperature.
6. The LPNs are washed three times using an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA) with a molecular weight cut-off of 10 kDa. The washed LPNs are re-suspended in water or buffer at a final desired concentration.
7. The LPNs are used immediately, stored at 4 °C overnight, or lyophilized for extended storage at –80 °C.

References

(1) Zhang, L.; Gu, F. X.; Chan, J. M.; Wang, A. Z.; Langer, R. S.; Farokhzad, O. C. *Clin. Pharmacol. Ther.* **2007**, *83* (5), 761–769.
(2) Sharma, A.; Sharma, U. S. *Int. J. Pharm.* **1997**, *154* (2), 123–140.
(3) Liechty, W.; Kryscio, D.; Slaughter, B.; Peppas, N. *Annu. Rev. Chem. Biomol. Eng.* **2010**, *1*, 149.
(4) Krishnamurthy, S.; Vaiyapuri, R.; Zhangb, L.; Chan, J. *Biomater. Sci.* **2015**, DOI: 10.1039/C4BM00427B
(5) Hasan, W.; Chu, K.; Gullapalli, A.; Dunn, S.; Enlow, E.; Luft, J. C.; Tian, S.; Napier, M., Pohlhaus, P.; Rolland, J.; DeSimone, J. *Nano Lett.* **2012**, *12* (1), 287–292.
(6) Zheng, Y.; Yu, B.; Weecharangsan, W.; Piao, L.; Darby, M.; Mao, Y.; Koynova, R.; Yang, X.; Li, H.; Xu, S.; Lee, L. J.; Sugimoto, Y.; Brueggemeier, R.; Lee, R. *Int. J. Pharm.* **2010**, *390* (2), 234–241.
(7) Gao, L. Y.; Liu, X. Y.; Chen, C. J.; Wang, J. C.; Feng, Q.; Yu, M. Z.; Ma, X. F.; Pei, X. W.; Niu, Y. J.; Qiu, C.; Pang, W. H.; Zhang, Q. *Biomaterials* **2014**, *35* (6), 2066–2078.
(8) Thevenot, J.; Troutier, A. L.; David, L.; Delair, T.; Ladavière, C. *Biomacromolecules* **2007**, *8* (11), 3651–3660.
(9) Zhang, L.; Chan, J.; Gu, F. X.; Rhee, J-W.; Wang, A.; Radovic-Moreno, A.; Alexis, F.; Langer, R.; Farokhzad, O. *ACS Nano* **2008**, *2* (8), 1696–1702.
(10) Aravind, A.; Jeyamohan, P.; Nair, R.; Veeranarayanan, S.; Nagaoka, Y.; Yoshida, Y.; Maekawa, T.; Kumar, S. *Biotechnol. Bioeng.* **2012**, *109* (11), 2920–2931.
(11) Clawson, C.; Ton, L.; Aryal, S.; Fu, V.; Esener, S.; Zhang, L. *Langmuir* **2011**, *27* (17), 10556–10561.
(12) Sengupta, S.; Eavarone, D.; Capila, I.; Zhao, G.; Watson, N.; Kiziltepe, T.; Sasisekharan, R. *Nature* **2005**, *436* (7050), 568–572.
(13) Liu, Y.; Li, K.; Pan, J.; Liu, B.; Feng, S-S. *Biomaterials* **2010**, *31* (2), 330–338.
(14) Wang, H.; Zhao, Y.; Wu, Y.; Hu, Y. L.; Nan, K.; Nie, G.; Chen, H. *Biomaterials* **2011**, *32* (32), 8281–8290.
(15) Kumar, S. S. D.; Mahesh, A.; Mahadevan, S.; Mandal, A. B. *Biochim. Biophys. Acta (BBA) - General Subjects* **2014**, *1840* (6), 1913–1922.
(16) Prasad, P.; Shuhendler, A.; Cai, P.; Rauth, A.; Wu, X. Y. *Cancer Lett.* **2013**, *334* (2), 263–273.
(17) Chan, J. M.; Zhang, L.; Yuet, K. P.; Liao, G.; Rhee, J. W.; Langer, R.; Farokhzad, O. C. *Biomaterials* **2009**, *30* (8), 1627–1634.

Lipids for Drug Delivery

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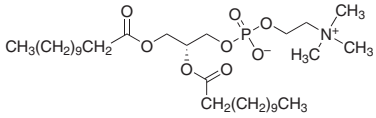
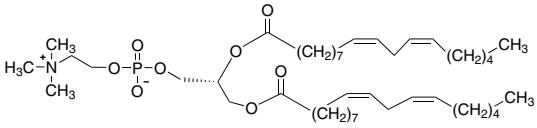
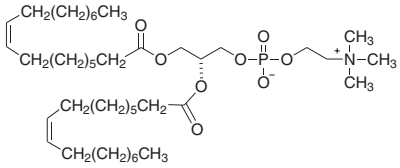
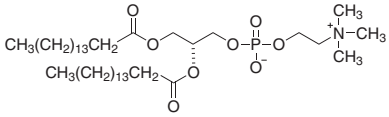
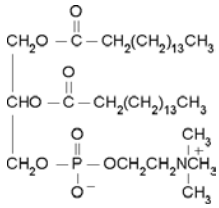
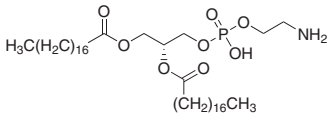
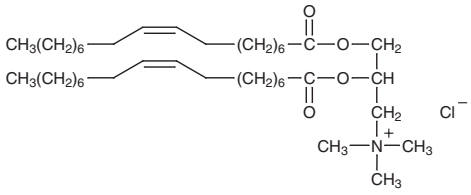
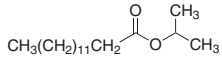
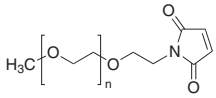
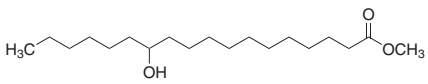
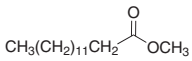
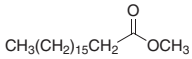
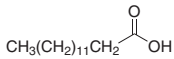
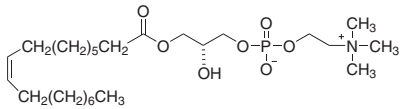
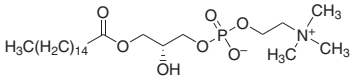
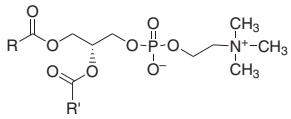
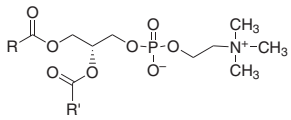
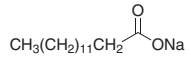
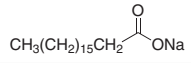
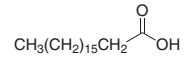
Product Description	Structure	Purity	Prod. No.
1,2-Didodecanoyl-*sn*-glycero-3-phosphocholine, synthetic		≥99%	P1263-25MG P1263-100MG P1263-500MG
1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine		≥99%, TLC	P0537-5MG P0537-25MG
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine		-	P6354-25MG P6354-100MG P6354-1G
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, semisynthetic		≥99%	P0763-50MG P0763-100MG P0763-250MG P0763-1G P0763-5G
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine		≥99%, TLC	P4329-25MG P4329-100MG P4329-500MG P4329-1G
1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine		~99%	P5911-100MG P5911-250MG P5911-1G
1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine		≥99%	P3531-100MG P3531-500MG
DOTAP chloride		≥98%, TLC	D6182-50MG D6182-250MG
Isopropyl myristate		≥90%, GC	M0757-250ML M0757-1L
L-α-Lysophosphatidylcholine	-	≥99%	L4129-25MG L4129-100MG L4129-500MG L4129-1G
	-	≥99%	L0906-25MG L0906-100MG L0906-500MG
Methoxypolyethylene glycol maleimide		≥90%, NMR	63187-1G 63187-5G

Product Description	Structure	Purity	Prod. No.
Methyl 12-hydroxystearate		≥99%, GC	H7002-1G
Methyl myristate		≥99%, GC	M3378-1G M3378-25G
Methyl stearate		~99%, GC	S5376-1G S5376-5G S5376-10G S5376-50G
Myristic acid		≥99%	M3128-10G M3128-100G M3128-500G
		≥98.0%, GC	70082-50G 70082-250G 70082-1KG
1-Oleoyl-*sn*-glycero-3-phosphocholine, synthetic		≥99%	L1881-5MG L1881-25MG L1881-100MG
1-Palmitoyl-*sn*-glycero-3-phosphocholine, synthetic		≥99%	L5254-25MG L5254-50MG L5254-100MG L5254-250MG L5254-1G
L-α-Phosphatidylcholine, egg yolk	 R, R' = fatty acid residues	≥99%, TLC	P3556-25MG P3556-100MG P3556-500MG P3556-1G
L-α-Phosphatidylcholine, from egg yolk		~60%, TLC	61755-25G 61755-100G
L-α-Phosphatidylcholine, dried egg yolk		≥40%, enzymatic	P5394-10G P5394-25G P5394-100G P5394-500G
L-α-Phosphatidylcholine, egg yolk		≥99%	P2772-100MG P2772-250MG P2772-500MG P2772-1G
		≥99%, TLC	P4279-100MG P4279-250MG P4279-1G
L-α-Phosphatidylcholine, soybean	 R, R' = fatty acid residues	≥99%, TLC	P7443-100MG P7443-500MG P7443-1G
		≥30%, enzymatic	P3644-25G P3644-100G P3644-500G P3644-1KG
		14-23% choline basis	P5638-500G P5638-1KG
L-α-Phosphatidylcholine, hydrogenated	-	≥99%	P4139-100MG P4139-1G
Sodium myristate		≥99%	M8005-10G M8005-25G
Sodium stearate		≥99%	S3381-1G S3381-5G S3381-25G
Stearic acid		≥98.5%, capillary GC	S4751-1G S4751-5G S4751-10G S4751-25G S4751-100G

Controlled Release | Targeted Delivery | Solubility Enhancement

CROSSLINKED CHITOSAN NANOPARTICLES
AND CHEMICAL MODIFICATIONS FOR DRUG DELIVERY APPLICATIONS

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Introduction

As a polysaccharide, chitosan can have a large density of reactive groups and a wide range of molecular weights (M_w). Chitosan is a linear heteropolymer of *N*-acetyl-D-glucosamine and D-glucosamine linked by β-(1–4)glycosidic bonds (**Figure 1**). It is obtained by partial deacetylation of chitin, the second largest and most abundant polysaccharide in nature after cellulose. The degree of acetylation (DA) is an essential characteristic of chitin and chitosan. It represents the fraction of *N*-acetyl-D-glucosamine relative to the total number of units.

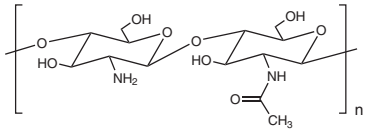


Figure 1. Chemical structure of chitosan

For many years chitosan was considered useful as a bioadhesive material because of its ability to form non-covalent bonds with biological tissues, mainly epithelia and mucous membranes. Bioadhesions formed using natural polymers have unique properties as a carrier because they can prolong residence time and, therefore, increase the absorbance of loaded drugs.[1] Chitosan is hydrophilic and soluble in acidic solutions through the protonation of its amine groups. Modified and unmodified chitosan has been widely used, albeit with different molecular weights and chemical modifications, in biomedical,[2,3] pharmaceutical,[4] metal chelation,[5,6] food additive,[7] and other industrial applications.[8] Chitosan is biocompatible and can be biodegraded by enzymes such as lysozymes, some lipases, and proteases.[9] These properties, as well as its positive charge in physiological conditions, endow chitosan with a promising future as a biomaterial.

Nanoparticle drug delivery systems, including nanospheres, nanocapsules, nanomicelles, nanoliposomes, etc., are nanometric carriers used to deliver drugs or biomolecules by trapping active agents in their interior structures and/or adsorbing them onto their exterior surfaces.[1,10–11] Presently, nanoparticles (NPs) have been widely used to deliver drugs, polypeptides, proteins, vaccines, genes, and nucleic acids. Recently, there has been increased interest in the use of NPs containing natural polysaccharides for drug delivery applications.[12–13] A large number of studies have been conducted on polysaccharides and their derivatives for their potential application as NP drug delivery systems, and chitosan has been identified among the most promising candidates.[14–16]

The following sections focus on the leading techniques for preparation and application of chitosan NPs, as well as chemical modification methods for self-assembly structures including nanoparticles and nanomicelles.

Chitosan NP Preparation Methods

Particulate chitosan structures are 3D crosslinked networks where polymeric chains are interconnected by crosslinkers. The main parameter, which determines the properties of a crosslinked NP, such as drug release and mechanical strength, is the crosslinking density.[17] Depending on the desired chitosan structural characteristics, nanoparticles are prepared mainly by four mechanisms:

1. Covalent crosslinking
2. Ionic crosslinking
3. Polyelectrolyte complexation
4. Self-assembly of hydrophobically modified polysaccharide

In general, for mechanisms 1–3, chitosan NP preparation starts with the dropwise addition of the desired crosslinker to the chitosan solution with continuous stirring for 1–24 h, with or without slight heating depending on the crosslinking chemistry. However, the fourth mechanism of NP preparation, in addition to hydrophobic moiety chemistry, depends on two parameters: the percentage of hydrophobic substitution (Degree of Substitutions—DS%) and the final modified chitosan concentration (lower for nanoparticles and nanomicelles; higher within hydrogels). **Table 1** summarizes and compares these four mechanisms as well as current delivery applications. **Figure 2** represents illustrative photos on the crosslinked chitosan 3D structure formation highlighting internal interactions. Hydrophobic groups on chitosan confer new physicochemical properties, including the ability to self-associate in water or under sonication,[18–19] to form different types of drug delivery systems (**Figure 2**).

Table 1. Crosslinked chitosan NP synthesis mechanisms and drug delivery applications—comparison study.

	Chitosan NP Formulation Mechanisms			
Mechanism Parameters	**Covalent Crosslinking**	**Ionic Crosslinking**	**Polyelectrolyte Complexation**	**Self-assembly of Hydrophobically Modified Chitosan**
Interaction	Irreversible chemical linkers	Reversible ionic crosslinking	Multi-ionic crosslinking	Intramolecular and/or intermolecular associations in water. Hydrophobic moieties interactions
Crosslinker/Moiety Characteristics	Two reactive functional groups (at least)	Small ionic molecules	Polyelectrolyte or larger ionic molecules	Hydrophobic nature
Main Interaction	Covalent bond interactions	Electrostatic interactions	Multiple electrostatic interactions	Hydrophobic interactions
Secondary Interactions	Hydrogen bonds and hydrophobic interactions	Hydrogen bonds	Hydrogen bonds	Hydrogen bonds
Classical Crosslinkers/ Hydrophobic Moiety	Dialdehydes: Glutaraldehyde Di/tricarboxylic acids	Tripolyphosphate (TPP)	Alginate, Heparin, Peptide, Nucleic acid, Poly(acrylic acid), Carboxymethyl Cellulose	Linoleic acid, Stearic acid, 5β-Cholanic acid, Deoxycholic, Oleoyl, Cholesterol
Structures	NPs	NPs	NPs	NPs and Nanomicelles
Biocompatibility and Toxicity	Aldehydes are highly toxic, while natural carboxylic-based crosslinkers are considered biocompatible alternatives	Enhanced biocompatibility since the preparation of NPs are by reversible ionic crosslinking, without use of aldehyde or toxic crosslinkers	Enhanced biocompatibility since the preparation of NPs are by reversible ionic crosslinking, without use of aldehyde or toxic crosslinkers	Related to modification moiety, particulate structure, and DS%
Particles Size	200–400 nm	100–350 nm	200–900 nm	80–1,400 nm
Drugs and Drug Delivery Applications	Toothpaste applications	Nanoparticles as carriers for the anticancer drug doxorubicin	Great potential in gene delivery: plasmid DNA as well as insulin and bovine serum albumin	Drug delivery to brain tumor or general anticancer drug delivery: methotrexate, paclitaxel, doxorubicin, epirubicin
References	20–22	20	17,20,23–25	26–28
Notes	Rigid network structures	Mild-rigid network structures	pH-responsive drug release network structures	NP size is controlled by adjusting the length of the hydrophobic moiety and chitosan M_w.[17] Core hydrophobic—optimal for hydrophobic drugs delivery. Prolonged circulation and thermodynamic stability.[29] Hydrogel could form at high concentration and DS% levels.

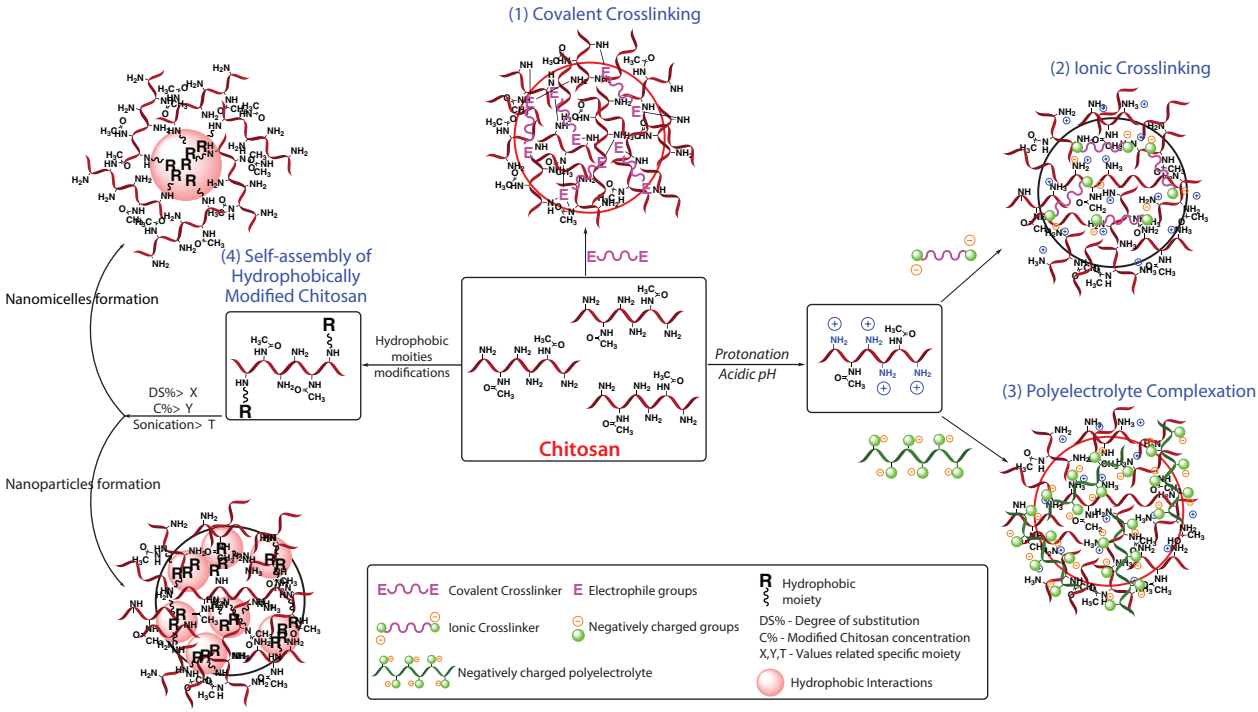


Figure 2. Mechanisms of chitosan nanoparticle formation and hydrophobically modified chitosan self-assembly nanomicelles.

Identification of the most appropriate mechanism for chitosan nanoparticle formation can be complicated. Many factors must be considered that may affect the rational design of nanoparticulate delivery systems, including drug nature and loading capacity, delivery duration, chitosan M_w, NP shape and size, targeting site, and biocompatibility. On the other hand, several factors also influence the crosslinking reaction such as the characteristics and chemical structure of the crosslinker itself. In covalent crosslinking, for example, the most crucial factor is the crosslinker size/length; in ionic crosslinking, the global charge of both the crosslinker and the polysaccharide are dominant. Additionally, unlike covalently crosslinked NPs, ionically crosslinked particles are generally pH sensitive, a desired trait for drug delivery purposes.

Chitosan Modifications: NPs and Nanomicelles

In addition to M_w diversity, chitosan has a variety of reactive groups, including hydroxyl and amino groups (**Figure 1**), which allow for the possibility of chemical modification to obtain amphiphilic properties.[1] This gives it a new or improved property. Chitosan, chemically modified through the grafting of hydrophobic groups, undergoes intra and/or inter-molecular hydrophobic interactions. Amphiphilic properties allow it to self-associate in aqueous solution, leading to different kinds of drug delivery systems such as nanomicelles, nanoparticles, microspheres,[30] liposomes,[31] and hydrogels (**Figure 2. Mechanism 4**). Modified chitosans are of great interest for the development of various controlled-release systems.

Since the amine group of chitosan is more reactive than the hydroxyl groups, all the research describing the formation of amphiphilic chitosan have been based on the chemical grafting of hydrophobic groups on the amine functional group by *N*-acylation reactions.[32] The following section reviews experimental procedures for the chemical modification of chitosan based on *N*-acylation and current applications in drug delivery systems, as listed in **Table 1** (self-assembly part) and **Table 2**.

Delivery of hydrophobic molecules and proteins has always been an issue due to poor bioavailability after administration. Micelle carrier systems can improve drug solubility and stability as well as help overcome toxicity and immunogenicity problems. It is well known the hydrophobic core of the micelles provides a reservoir for loading water-insoluble drugs. By grafting hydrophobic moieties to the polysaccharide backbone, self-assembled micelles can be readily formed in aqueous solution. Aggregation of amphiphilic polymers is controlled by the balance between the interaction of the hydrophobic groups and the hydrophilic chains. The critical aggregation concentration (CAC) is the concentration at which the polymer aggregation starts.

It was found that a chitosan EDC-mediated modification with stearic acid, linoleic acid, deoxycholic acid, and 5β-cholanic acid tends to self-aggregate to form nanomicelles at very low CAC (0.01–0.06 mg/mL).[33–36] Amphiphilic chitosan-based micelles were used to encapsulate doxorubicin,[37] paclitaxel,[27,34,38] ibuprofen,[39] and the amphiphilic adriamycin.[40] Furthermore, hydrophilic peptides, proteins, and nucleic acids[41,42] can be adsorbed onto chitosan-based micelles.

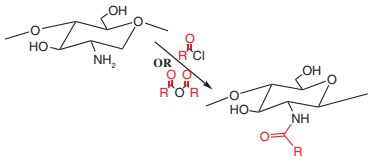
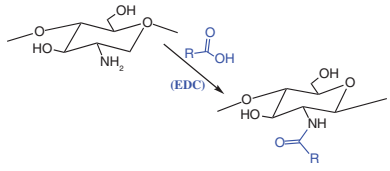
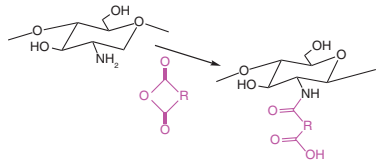
Concluding Remarks

This short review summarizes the recent research on chitosan and its amphiphilic derivatives, including experimental chemical modifications, the mechanisms of nano-structure fabrication, and their drug delivery system applications. Special attention is increasingly focused on modified chitosan with amphiphilic properties because of unique properties such as excellent biocompatibility and biodegradability, non-toxicity, and bioadhesive properties. Self-assembly of amphiphilic chitosan provides further promise for drug delivery systems since several properties such as size, surface charge, loading efficiency, stability, and biodistribution can be altered for a particular application. Chitosan-based nanomicelle systems have been found to improve delivery of hydrophobic drugs and proteins, increase stability, and exhibit controllable drug release properties, biocompatibility, and improved targeting for specific applications—all of which are of increasing interest for clinical application.

References

(1) Liu, Z. et al., *Adv. Drug Deliv. Rev.* **2008**, *60*, 1650–1662.
(2) Langer, R.; Tirrell, D. A. *Nature* **2004**, *428*, 487–492.
(3) Alvarez, N. M.; Mano, J. F. *Int. J. Biol. Macromol.* **2008**, *43*, 401–414.
(4) Pedro, A. S. et al. *Carbohydr. Polym.* **2009**, *76*, 501–508.
(5) Guibal, E. et al. *Int. J. Biol. Macromol.* **2001**, *28*, 401–408.
(6) Schmuhl, R. et al. *Water SA* **2001**, *27*, 1–7.
(7) Shahidi, F. et al. *Trends Food Sci. Technol.* **1999**, *10*, 37–51.
(8) Majeti, N. V.; Kumar, R. *React. Funct. Polym.* **2000**, *46*, 1–27.
(9) Bardot, P.M.; et al. *Presses universitaires de Franche-Comte´* mars **2009**. *308*, ISBN: 2-84867-249-8.
(10) Jung, T. et al. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 147–160.
(11) Eliaz, R. E.; Szoka, F.C. *Cancer Res.* **2001**, *61*, 2592–2601.
(12) Coviello, T. et al. *J. Controlled Release* **2007**, *119*, 5–24.
(13) Vauthier, C.; Bouchemal, K. *Pharm. Res.* **2009**, *26*, 1025–1058.
(14) Sinha, V. R.; Kumria, R. *Int. J. Pharm.* **2001**, *224*, 19–38.
(15) Vandamme, T. F. et al. *Carbohydr. Polym.* **2002**, *48*, 219–231.
(16) Lemarchand, C. et al. *Eur. J. Pharm. Biopharm.* **2004**, *58*, 327–341.
(17) Mizrahy, S.; Peer, D. *Chem. Soc. Rev.* **2012**, *41*, 2623–2640.
(18) Liu, C. et al. *J. Ocean Univ. China* **2005**, *4*, 234–239.
(19) Liu, C. et al. *Curr. Appl. Phys.* **2007**, *7*, 125–129.
(20) Berger, J. et al. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 19–34.
(21) Liu, H. et al. *J. Appl. Polym. Sci.* **2007**, *106*, 4248–4256.
(22) Bodnar, M. et al. *Biomacromolecules* **2005**, *6*, 2521–2527.
(23) Hamman, J. H. *Mar. Drugs* **2010**, *8*, 1305–1322.
(24) Park, J. H. et al. *Adv. Drug Delivery Rev.* **2010**, *62*, 28–41.
(25) Sajeesh, S.; Sharma, C.P. *J. Biomed. Mater. Res.* **2006**, *76B*, 298–305.
(26) Yang, X. D. et al. *Colloids Surf., B* **2008**, *61*, 125–131.
(27) Hu, F. Q. et al. *Colloids Surf., B* **2006**, *50*, 97–103.

Table 2. Chitosan modifications—experimental reactions and applications

Modification Parameters	Chitosan *N*-acylation Modifications via:		
	***N*-acetyl Chloride**	***N*-acetyl Carboxylic Acid**	***N*-carboxyacyl**
Chemical Reaction (Amino Repeating Unit in Chitosan)			
Hydrophobic Moiety	Oleoyl chloride[28]	Stearic acid ($C_{18}H_{36}O_2$)[27,45] Linoleic acid ($C_{18}H_{32}O_2$)[46] Oleic acid ($C_{18}H_{34}O_2$)[47] Deoxycholic acid[35,41]	Cyclic acid anhydride (R): acetic, propionic, *n*-butyric, *n*-valeric, hexanoic, octanoic, lauric, palmitic, and stearic
Reaction Conditions	In mixture (pyridine/chloroform) with oleoyl chloride at room temperature for 2 h, followed by reflux for 10 h. Purification: Crude product was poured into methanol, filtered and dried under vacuum for 24 h.[43]	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).	Reactions performed in presence of dimethyl sulfoxide.[48–49]
Notes	DS calculation by infrared spectroscopy, ratio of absorbance at 1,655 cm^{-1} (amide I band) and the hydroxyl band at 3,450 cm^{-1}.[44]	NP size increased with the amount of active ingredient encapsulated.	
Drug Delivery Applications	NPs as carriers for anticancer drug doxorubicin.	For linoleic acid: magnetic resonance imaging of the liver. NP carriers for anticancer drugs: paclitaxel and doxorubicin.	NP carriers for anticancer drugs

(28) Zhang, J. et al. *Nanomed-Nanotechnol.* **2007**, *3*, 258–265.
(29) Letchford, K.; Burt, H. *Eur. J. Pharm. Biopharm.* **2007**, *65*, 259–269.
(30) Mi, F. L. et al. *Carbohydr. Polym.* **2005**, *60*, 219–227.
(31) Liang, G. et al. *J. Pharm. Pharmacol.* **2007**, *59*, 661–667.
(32) Hassani, L. N. et al. *Drug Discovery Today* **2012**, *17*, Num. 11/12.
(33) Kwon, S. et al. *Langmuir* **2003**, *19*, 10188–10193.
(34) Kim, J. H. et al. (Reprinted from *J. Controlled Release* **2005**, *109*, 1), *J. Controlled Release* **2006**, *111*, 228–234.
(35) Lee, K. Y. et al. *Macromolecules* **1998**, *31*, 378–383.
(36) Kim, K. et al. *Macromol. Res.* **2005**, *13*, 167-175.
(37) Ye, Y. Q. et al. *Int. J. Pharm.* **2008**, *352*, 294–301.
(38) Zhang, Y. et al. *Carbohydr. Polym.* **2009**, *77*, 231–238.
(39) Jiang, G. B. et al. *Mol. Pharm.* **2006**, *3*, 152–160.
(40) Lee, K. Y. et al. *Colloid. Polym. Sci.* **2000**, *278*, 1216–1219.
(41) Lee, K. Y. et al. *J. Controlled Release* **1998**, *51*, 213–220.
(42) Lee, K. Y. et al. *Polymer* **2005**, *46*, 8107–8112.
(43) Li, Y. Y. et al. *J. Appl. Polym. Sci.* **2006**, *102*, 1968–1973.
(44) Le Tien, C. et al. *J. Controlled Release* **2003**, *93*, 1–13.
(45) Hu, F. Q. et al. *Biomaterials* **2009**, *30*, 6955–6963.
(46) Liu, C. G. et al. *Carbohydr. Polym.* **2005**, *62*, 293–298.
(47) Huang, L. et al. *Front. Biol. China* **2009**, *4*, 321–327.
(48) Lee, M. Y. et al. *Int. J. Biol. Macromol.* **2005**, *36*, 152–158.
(49) Mourya, V. K.; Inamdar, N. N. *React. Funct. Polym.* **2008**, *68*, 1013–1051.

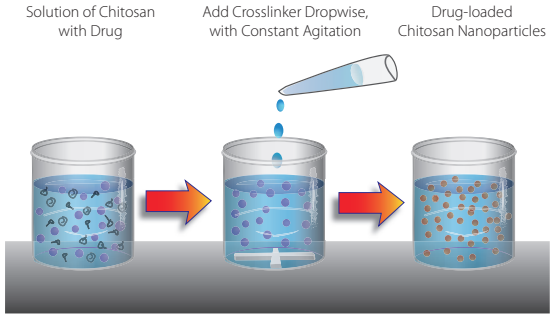
TECHNICAL SPOTLIGHT

Chitosan Nanoparticles by Covalent or Ionic Crosslinking

Chitosan nanoparticles can be formed by a variety of methods including emulsion, ionic gelation, reverse micellar methods, or self-assembly. The example method given here uses the simplest approach to form either chemical or ionic crosslinked chitosan nanoparticles. This method utilizes dropwise addition of a crosslinker to a solution of chitosan under constant stirring.

1. Prepare chitosan solution: Dissolve chitosan at 0.1–1 wt% in 1–3% acetic acid, once dissolved adjust pH to 4.7.
2. Prepare crosslinker solution:
   a) For Ionic gelation: dissolve crosslinker (Tripolyphosphate (TPP), dextran sulphate, etc.) in deionized water at 0.1 wt% to 0.5 wt%.
   b) For covalent crosslinking: adjust crosslinker concentration (example: 1–3% dialdehyde or di/tricarboxylic acid).
3. Slowly add crosslinker solution (1 mL) dropwise into Chitosan solution (3 mL) at room temperature with constant stirring.
4. Allow nanoparticles to stabilize by a 30 min incubation at room temperature.
5. Collect nanoparticles by centrifugation at 13,000 × *g* at 10 °C for 30 min, and resuspend nanoparticles in aqueous solution.
6. To incorporate drug, add drug to crosslinker aqueous solution prior to addition to the chitosan solution.

Note: Chitosan and crosslinker concentration need to be optimized for desired particle size and encapsulation efficiency.



Natural Polymers

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Name	Inherent Viscosity (cP)	Degree of Deacetylation	Prod. No.
Chitosan	20-300 (1 wt. % in 1% acetic acid Brookfield)	75-85% deacetylated	448869-50G 448869-250G
	200-800 (1 wt. % in 1% acetic acid Brookfield)	75-85% deacetylated	448877-50G 448877-250G
	800-2000 (1 wt. % in 1% acetic acid Brookfield)	>75% deacetylated	419419-50G 419419-250G
Alginic acid	15-20 1 % in H_2O	-	180947-100G 180947-250G 180947-500G

Controlled Release | Targeted Delivery | Solubility Enhancement

POLY(N-ISOPROPYLACRYLAMIDE)-BASED STIMULI-RESPONSIVE MATERIALS


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Introduction

Controlled delivery of therapeutic agents has generated significant interest[1–3] because it can improve drug efficacy by preventing premature degradation, enhance uptake, reduce side effects, and help to maintain appropriate therapeutic concentration in the bloodstream. Significant research effort has been devoted to the development of systems that can deliver defined quantities of a therapeutic payload in a site-specific and/or time-controlled fashion. Devices made with stimuli-responsive materials have attracted considerable interest for use in controlled delivery.[4–6] Stimuli-responsive or "smart" materials undergo dramatic property changes in response to small changes in the environment and can be used as programmable, responsive, and adaptive materials. The response may be produced through artificial stimuli such as thermal, light-irradiation, magnetic, ultrasonic, and electric, as well as natural stimuli like changes in pH, ionic strength, redox gradients, and enzymatic stimuli.[4] The use of stimuli-responsive delivery devices offers an interesting opportunity for controlled delivery where the delivery system becomes an active participant rather than a passive vehicle in the optimization of a therapy.[7] The benefit of stimuli responsive nano-carriers is especially important when the stimuli are unique to disease pathology, allowing the nano-carrier to respond specifically to the pathological "triggers" such as pH, temperature, and redox microenvironment.

Poly(N-isopropylacrylamide): A Temperature-sensitive Polymer

Among the stimuli-responsive materials, thermo- and pH-sensitive materials are the most frequently used for controlled drug delivery because of the different thermal and pH conditions within various tissue and cellular compartments along the endocytic pathway.[8,9] For example, tumor tissues have slightly higher temperature and lower pH than healthy tissues. These variations in temperature and pH can be exploited for the targeted release of payloads at specific sites.[9] Stimuli-responsive drug delivery platforms often are prepared by formulating or functionalizing the system with thermo- and/or pH-sensitive polymeric materials.[10] These materials have polymer–polymer and polymer–solvent interactions that change abruptly with a small change in pH and/or temperature, which translates to a polymer chain transition between extended and compacted coil states. In drug delivery, this chain configuration disrupts the integrity of the delivery vehicle and triggers release of the drug.

Poly(N-isopropylacrylamide) (PNIPAM) (**Figure 1**) is a unique hydrophilic polymer with a stimuli-responsive transition temperature close to physiological temperature.

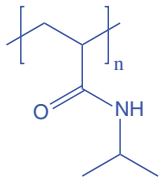

Figure 1. Structure of PNIPAM.

In aqueous solution, PNIPAM exhibits a thermo-responsive phase transition at 32 °C. This transition temperature is called the lower critical solution temperature (LCST). Below the LCST, PNIPAM is water-soluble and hydrophilic, with an extended chain conformation. At LCST, PNIPAM undergoes a phase transition to a hydrophobic aggregate state becoming water insoluble above the LCST. This phase transition occurs within a remarkably narrow temperature range and is reversible. The macroscopic manifestation of this thermal change depends on the chain configuration (**Figure 2**).[11]

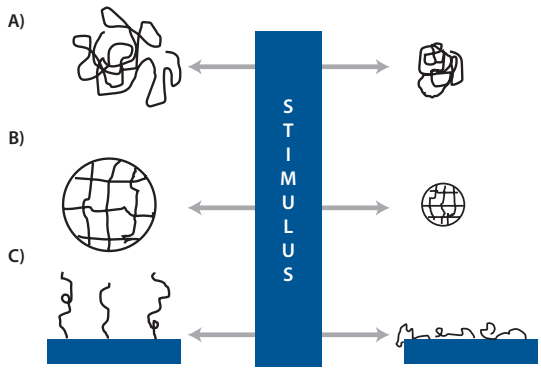

Figure 2. Classification of stimuli-responsive polymers by their physical form and their response to external stimuli. **A**) Linear free chains in solution will undergo a reversible collapse after stimulus is applied. **B**) Covalently crosslinked reversible gels where swelling or shrinking of the gels can be triggered by environmental change. **C**) Chain adsorbed or surface-grafted form, where the polymer reversibly swells or collapses on the surface once an external parameter is changed.[11]

The solubility of PNIPAMs below the LCST and lack of functional groups for further modification can limit the utility of the unmodified polymer in many drug delivery applications.[12] Therefore, PNIPAM is often modified to tune the LCST through the synthesis of copolymers using hydrophilic or hydrophobic monomers. For example, when NIPAAm is copolymerized with the hydrophilic monomers acrylamide (AAm), the LCST increases to 45 °C with an AAm content of 18%. Conversely, copolymers can be synthesized with an LCST of 10 °C when 40% of the hydrophobic *N-tert*-butylacrylamide (N-tBAAm) monomer is added to the polymer.[12–14] Copolymers with both thermal and pH sensitivity have been synthesized with acidic monomers such as acrylic acid or methacrylic acid.[16–21] In addition, protein and peptide conjugates of PNIPAM and copolymers of poly(NIPAM-co-acrylic acid) have been explored to further modify the LCST or stimuli responsiveness.[22–24]

PNIPAM Micelles

Amphiphilic copolymers containing PNIPAM have been used to form nano-assembled aggregates for use as drug delivery carriers.[25–27] To do this, block copolymers consisting of a PNIPAM segment and a hydrophobic segment are used to form core–shell micellar structures below the PNIPAM LCST. The inner hydrophobic core is then loaded with water-insoluble drugs; the PNIPAM outer shell imparts temperature responsiveness and aqueous solubilization. Upon reaching the LCST, nano-assemblies of PNIPAM-based micelles loaded with active ingredients collapse and release the payload (**Figure 3**).[28,29] Chung et al. demonstrated enhanced drug release in response to temperature fluctuations and improved cytotoxicity of Doxorubicin to cancer cells when Poly(N-isopropylacrylamide-b-butylmethacrylate) micelles were used as a drug carrier.[30]

PNIPAM Drug Conjugates

Stimuli-responsive polymers have also been covalently linked to biologics for controlled and targeted drug delivery. Polymer bioconjugates can improve aqueous stability, reduce immunogenenicity, minimize toxicity, and increase *in vivo* circulation times of biological drugs.[31–33] Site-specific delivery may be obtained by tailoring the conjugates as an inactive prodrug and designing polymer drug linkages susceptible to cleavage by specific enzymes or pH.[34,35] Bioconjugation is generally accomplished using polymers with chemically reactive end-functional groups.

PNIPAMs have been developed with a range of functional groups including amine,[36–38] carboxylic,[39–41] NHS ester,[42,43] and thiol.[44,45] Recent developments in controlled radical polymerization, such as RAFT, provide a very simple tool to prepare end-functionalized PNIPAM with well-defined structures[46–48] that can be used to make nanocarriers amiable to bioconjugation.[49–51] Hoffman et al. prepared amine-terminated PNIPAMs using the amine for protein conjugation.[52] Meyer et al.[39] demonstrated enhanced and targeted delivery of therapeutics to solid tumors with an amine-terminated poly(NIPA-co-AAm)/rhodamine conjugate. Furthermore, carboxyl-terminated PNIPAM has been used for bioconjugation.[53–55]

PNIPAM-coated Liposomes

PNIPAM can also be incorporated into liposomes for drug delivery by modifying the PNIPAM polymer structure. The incorporation of long hydrocarbon chains such as C-18 (either on the end or randomly along the PNIPAM backbone) enables polymer anchoring into the liposome bilayer.[56–58] The resulting PNIPAM coated liposomes act as stimuli-sensitive delivery devices for tumor delivery.[59,60] Moreover, liposomes were surface modified with PNIPAM copolymers, such as poly(N-isopropylacrylamide-co-acrylamide) (PNIPAM-AAM) and PEG.[61] The release of DOX from these PNIPAM-AAM/PEG-modified liposomes increases near the polymer transition temperature. In addition, these modified liposomes were found to be stable in serum compared with unmodified liposomes, indicating promise for use in targeted drug delivery.

Summary

Stimuli-responsive polymers, like PNIPAM, play an important role in designing advanced active drug delivery devices. Responsive polymers enable targeted and controlled drug delivery, resulting in superior pharmacokinetics. With advances in polymerization techniques, an abundance of PNIPAM copolymer variants can be made to produce materials with a wide range of physical and chemical properties and stimuli-responsive sensitivities. To date, numerous publications illustrate the potential of stimuli-responsive nanocarriers in the future arsenal of pharmaceutical formulations.

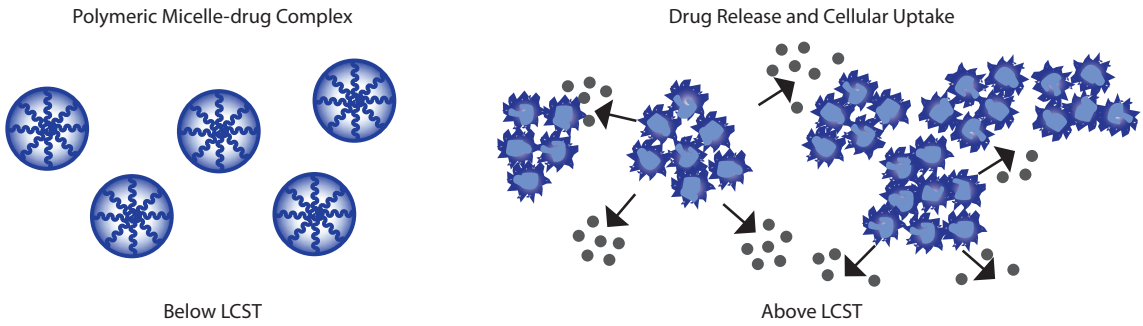

Figure 3. Schematic showing PNIPAM-based micelles. The drug is released at temperatures above the LCST.

Methods: PNIPAM Drug Delivery Systems

PNIPAM Micelles

Temperature-responsive drug-loaded micelles can be formed from amphiphilic polymers containing PNIPAM blocks.[46] The easiest method, dialysis, can be accomplished by first dissolving the copolymer at a dilute concentration in organic solvent (for example: 30 mg of PNIPAM copolymer in 5 mL of DMF or THF, where the organic solvent is selected based on the copolymer composition and solubility).

For drug encapsulation, the drug is added to the organic solvent prior to dialysis. The solution is dialyzed with a molecular weight cut-off membrane of 3,500 Da against ddH_2O for up to 5 days, depending on the frequency of water change.

PNIPAM copolymer micelles can be formed by additional techniques (highlighted by Du and Stenzel within this guide).

PNIPAM Protein Conjugation

As an example, amine-terminated PNIPAM (**Aldrich Prod. No.** 724823) can be used for protein conjugation or further modified to have a maleimide functional group for thiol conjugation.[52] For thiol conjugation, PNIPAM-amine is converted to PNIPAM-Mal, followed by conjugation to the protein therapeutic (**Figure 4**).

1. Synthesis of PNIPAM-Mal
   The amino end group is reacted with succinimidyl4-(N-maleimido-methyl)cyclohexane-1-carboxylate (**Sigma-Aldrich Prod. No.** M5525) in dichloromethane at a 1:1.5 ratio, resulting in PNIPAM-Mal. After the insolubles are filtered, the product can be isolated and purified by precipitation into 15× excess of diethyl ether.
2. Protein Conjugation
   a) *Prepare protein solution:* Dissolve the protein in a suitable aqueous buffer (for example, 1 mM protein in 50 mM phosphate, 1 mM EDTA buffer, pH 8.0), and reduce the thiol by using 1 mM dithiothreitol (DTT, **Sigma-Aldrich Prod. No.** D9779) for 10 min at 4 °C. Remove DTT by spin filtration or by passing the solution over a Sephadex® G-25 gel filtration column equilibrated with the starting buffer.
   b) *For protein conjugation:* mix protein solution from Step 2a with purified PNIPAM-Mal from Step 1 using a molar excess of PNIPAM-Mal (10× is recommended). Allow the reaction to proceed at room temperature for 4 h with gentle shaking. The resulting protein-PNIPAM conjugate can be purified by precipitation with 10% (v/v) saturated $(NH_4)_2SO_4$ and heating the solution to 37 °C. The precipitate is then separated by centrifugation at 10,000 g.

Note: purification can also be completed by additional techniques, such as column chromatography, depending on the nature of the protein.

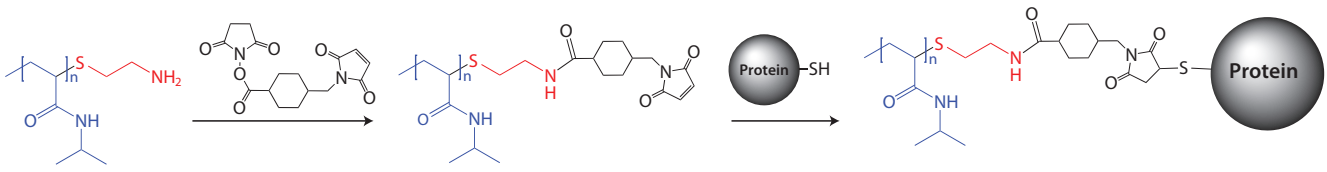

Figure 4. Scheme for PNIPAM maleimide conjugation to a protein-free thiol.

Preparation of PNIPAM-copolymer Coated Liposomes

Liposomes can be coated with a thermo- and pH-sensitive PNIPAM polymer to create stimuli-responsive drug delivery carriers.[59] For example, Poly(N-isopropylacrylamide-co-methacrylic acid-co-octadecyl acrylate) (**Aldrich Prod. No.** 724475), shown in **Figure 5**, contains hydrophobic octadecyl side chains randomly distributed along the polymer chain; the acid group imparts pH sensitivity to the polymer and octadecyl chain and helps anchor it to the lipid bilayer of the liposome.

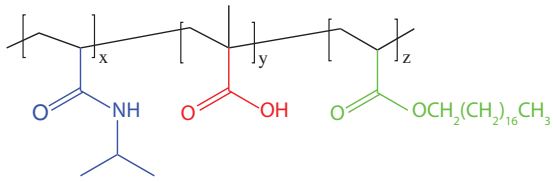

Figure 5. Structure of Poly(N-isopropylacrylamide-co-methacrylic acid-co-octadecyl acrylate).

As a general method, liposomes are first prepared by lipid hydration followed by extrusion, and the PNIPAM copolymer is added during the rehydration step. Liposome preparation involves three steps: vesicle formation, vesicle size reduction, and purification. The most common method for liposome preparation is lipid hydration and organic solvent exchange by reverse-phase evaporation or organic-solvent injection.

Preparation of Liposome

1. Dissolve 20 mg the lipid mixture of 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (**Sigma-Aldrich Prod. No.** 42773) and cholesterol (3:2 mol/mol) (**Sigma-Aldrich Prod. No.** C8667) in 10 mL of chloroform in a 100 mL flask. After lipid mixture is dissolved, remove chloroform at reduced pressure at 35 °C using rotary evaporation. After solvent is removed, continue rotary evaporation at high vacuum at least for 2 more hours to remove any remaining trace chloroform. This results in a dried lipid film around the inner wall of the flask.
2. Prepare drug-loaded liposomes
   a) For hydrophilic molecules:
      i. Dissolve drug in a suitable aqueous buffer.
      ii. Hydrate the dried lipid film by adding 5–10 mL of drug solution. Seal flask and sonicate for 3–5 min to form a lipid-drug emulsion.
   b) For hydrophobic drugs:
      iii. Dissolve drug in minimal quantity (5–10 mL) of an appropriate organic solvent (preferably low boiling solvent such as diethyl ether). Ensure drug is completely dissolved.
      iv. Add drug solution to dried lipid film formed in Step 1; mix well by sonication.
      v. To the lipid–drug solution, rapidly inject 1 mL of an aqueous solution (e.g., 150 mM NaCl, 0.02% w/w NaN_3 or another suitable buffer) using a 5 mL syringe.
      vi. Seal the flask and sonicate for 3 min to form a lipid–drug emulsion.
      vii. Remove the organic solvents by rotary evaporation under vacuum at 37 °C until a viscous gel layer is formed.
      viii. Agitate the gel vigorously using a vortex mixer to convert into a suspension of large unilamellar vesicles (LUVs).
3. Size reduction: liposome size can be decreased by using an extrusion system (LiposoFast) with a 0.2 µm pore size polycarbonate membrane. Membranes of different pore sizes may be used to obtain liposomes of the desired size range. After extrusion, remove non-encapsulated drugs by gel filtration (e.g., by using a Sephadex® G-100 gel column equilibrated with the aqueous buffer used in the liposome preparation stage above) or by spin-column filtration.
4. To create PNIPAM-coated liposomes:
   a) Prepare a 6 mg/mL PNIPAM stock solution of **Aldrich Prod. No.** 724475 in 150 mM NaCl and 0.02% w/w NaN_3 (or another suitable aqueous buffer for liposome rehydration).
   b) Add PNIPAM solution to drug-loaded liposome solution from Step 3.
   c) PNIPAM-coated liposomes can be stored in aqueous buffer at 4 °C or lyophilized.

References

(1) Zhang, Y.; Chan, H. F.; Kam W.; Leong, K. W. *Adv Drug Deliv Rev.* **2013**, *65*, 104.
(2) Rokstad, A. M. A.; Lacík, I.; de Vos, P.; Strand, B. L. *Adv Drug Deliv Rev.* **2014**, *67–68*, 111.
(3) Reddy, L. H.; Bazile, D. *Adv Drug Deliv Rev.* **2014**, *71*, 34.
(4) Fleige, E.; Quadir, M. A.; Haag, R. *Adv Drug Deliv Rev.* **2012**, *64*, 866.
(5) Shim, M. S.; Kwon, Y. J. *Adv Drug Deliv Rev.* **2012**, *64*, 1046.
(6) Meng, F.; Zhong, Z.; Feijen, J. *Biomacromolecules* **2009**, *10* (2), 197–209
(7) Ganta, S.; Devalapally,H.; Shahiwala, A.; Amiji, M. *J. Controlled Release* **2008**, *126*, 187.
(8) Schmaljohann, D. *Adv Drug Deliv Rev* **2006**, *58*, 1655.
(9) Yue Huang,Y.; Tang, Z.; Zhang, X.; Yu, H.; Sun, H.; Pang, X.; Chen, X. *Biomacromolecules* **2013**, *14*, 2023.
(10) Wei, H.; Cheng, S. X.; Zhang, X. Z.; Zhuo, R. X. *Prog. Polym. Sci.* **2009**, *34*, 893.
(11) Kumar, A.; Srivastava, A.; Galaev, I. Y.; Mattiasson, B. *Prog. Polym. Sci.* **2007,** *32*, 1205.
(12) Zintchenko, A.; Ogris, M.; Wagner, E. *Bioconjugate Chem.* **2006**, *17*, 766.
(13) Hoffman, A. S.; Stayton, P. S.; Bulmus, V. *J Biomed Mater Res* **2000**, *52*, 577.
(14) Okano, T.; Bae, Y. H.; Jacobs, H.; Kim, S. W. *J. Controlled Release* **1990**, *11*, 255.
(15) Yoshida, R.; Sakai, K.; Okano. T.; Sakurai, Y. *J Biomater Sci Polym Ed* **1994**, *75*, 55.
(16) Salgado-Rodrıguez, R.; Licea-Claverıe, A.; Arndt, K. F. *European Polymer Journal* **2004**, *40*, 1931.
(17) Schmaljohann, D. *Adv Drug Deliv Rev.* **2006**, *58*, 1655.
(18) Ringsdorf, H.; Venzmer, J.; Winnik, F. M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 315.
(19) Jones, M. S. *Eur Polym J* **1999**, *35*, 795.
(20) Bulmus, V.; Patır, S.; Tuncel, A.; Piskin, E. *J Appl Polym Sci* **2003**, *88*, 2012.
(21) Velada, J. L.; Liu, Y.; Huglin, M. B. *Macromol Chem Phys* **1998**, *199*, 1127.
(22) Bulmus, V.; Patır, S.; Tuncel, A.; Piskin, E. *J. Controlled Release* **2001**, *76*, 265.
(23) Chilkoti, A.; Chen, G.; Stayton, P. S.; Hoffman, A. S. *Bioconjugate Chem* **1994**, *5*, 504.
(24) Chen, G.; Hoffman, A. S. *Macromol Rapid Commun* **1995**, *16*, 175.
(25) Smith, A. E.; Xu, X.; McCormick, C. L. *Prog. Polym. Sci* **2010**, *35*, 45.
(26) Topp, M. D. C.; Dijkstra, P. J.; Talsma, H.; Feijen, J. *Macromolecules* **1997**, *30*, 8518.
(27) Hang, C, Y.; You, Y. Z.; Pan, C. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2004**, *42*, 4873.
(28) Meng, F.; Zhong, Z.; Feijen, J. *Biomacromolecules* **2009**, *10*, 197.
(29) Rijcken, C. J. F.; Soga, O.; Hennink, W. E.; Nostrum, C. F. V. *J. Controlled Release* **2007**, *120*, 131.
(30) Chung, J. E.; Yokoyama, M.; Yamato, M.; Aoyagi, T.; Sakurai, Y.; Okano, T. *J. Controlled Release* **1999**, *62*, 115.
(31) Duncan, R. *www.nature.com/reviews/cancer;* **2006**, *6*, 688.
(32) Vicent, M. J.; Dieudonné, L.; Carbajo, R. J.; Pineda-Lucena, A. *Expert Opin. Drug Deliv.* **2008**, *5*, 593.
(33) Meyer, O.; Papahadjopoulos, D.; Leroux, J. C. *FEBS Lett.* **1998**, *421*, 61.
(34) Hoffman, A. S.; Stayton, P. S. *Prog. Polym. Sci.* **2007**, *32*, 922.
(35) Lutz, J. F.; Börner, H. G. *Prog. Polym. Sci* **2008**, *33*, 1.
(36) Meyer, D. E.; Shin, B. C.; Kong, G. A.; Dewhirst, M. W.; Chilkoti, A. *J. Controlled Release* **2001**, *74*, 213.
(37) Wei, H.; Zhang, X. Z.; Cheng, H.; Chen, W. Q.; Cheng, S. X.; Zhuo, R. X. *J. Controlled Release* **2006**, *116*, 266.
(38) Postma, A.; Davis, T. P.; Li, G.; Moad, G.; O'Shea, M. S. *Macromolecules* **2006**, *39*, 5307.
(39) Konak, C.; Reschel, T.; Oupicky, D.; Ulbrich, K. *Langmuir* **2002**, *18*, 8217.
(40) Smithenry, D. W.; Kang, M. S.; Gupta, V. K. *Macromolecules* **2001**, *34*, 8503.
(41) Yasui, M.; Shiroya, T.; Fujimoto, K.; Kawaguchi, H. *Colloids and Surfaces B: Biointerfaces* **1997**, *8*, 311.
(42) Chen, X.; Ding, X.; Zheng, Z.; Peng, Y. *New J. Chem.* **2006**, *30*, 577–58.
(43) Yamazaki, A.; Song, J. M.; Winnik, F. M.; Brash, J. L. *Macromolecules* **1998**, *31*, 109.
(44) Scales, C. W.; Convertine, A. J.; McCormick, C. L. *Biomacromolecules* **2006**, *7*, 1389.
(45) Choi, S.; Choi, B. C.; Xue, C.; Leckband, D. *Biomacromolecules* **2013**, *14*, 92.
(46) Smith, A. E.; Xu, X.; McCormick, C. L. *Prog. Polym. Sci.* **2010**, *35*, 45.
(47) Henry, C. M.; Convertine, A. J.; Benoit, D. W.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2009**, *20*, 1122.
(48) York, A. W.; Kirkland, S. E.; McCormick, C. L. *Adv Drug Deliv Rev.* **2008**, *60*, 1018.
(49) Zhu, J. L.; Zhang, X. Z.; Cheng, H.; Li, Y. Y.; Cheng. S. X.; Zhuo. R. X. *J Polym Sci Polym Chem.* **2007**, *45*, 5354.
(50) Yan, J. J.; Ji, W. X.; Chen, E. Q.; Li, Z. C.; Liang, D. H. *Macromolecules* **2008**, *41*, 4908.
(51) You, Y. Z.; Oupicky, D. *Biomacromolecules* **2007**, *8*, 98.
(52) Chilkoti, A.; Chen, G.; Stayton, P. S.; Hoffman, A. S. *Bioconjugate Chem.* **1994**, *5*, 504.
(53) Chen, G.; Hoffman, A. S. *Bioconjugate Chem.* **1993**, *4*, 509.
(54) Chen, J-P.; Chu, D-H.; Sun Y-M. *J Chem Tech Biotechnol.* **1997**, *69*, 421.
(55) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. *Bioconjugate Chem.* **1993**, *3*, 42.
(56) Konoa, K.; Hayashib, H.; Takagishi, T. *J. Controlled Release* **1994**, *30*, 69.
(57) Polozova, A.; Winnik, F. M. *Biochimica et Biophysica Acta – Biomembranes* **1997**, *1326*, 213.
(58) Ringsdorf, H.; Sackmann, E.; Simon, J.; Winnik, F. M. *Biochimica et Biophysica Acta - Biomembranes* **1993**, *1153*, 335.
(59) Francis, M. F.; Dhara, G.; Winnik, F. M.; Leroux, J-C. *Biomacromolecules* **2001**, *2*, 741.
(60) Leroux, J-C.; Roux, E.; Garrec, D. L.; Hong, K.; Drummond, D. C. *J. Controlled Release* **2001**, *72*, 71.
(61) Han, H. D.; Shin, B. C.; Choi, H. S. *Eur.J. Pharm. Biopharm.* **2006**, *62*, 110.

Poly(N-isopropylacrylamide) (PNIPAM)

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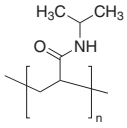
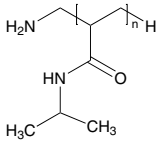
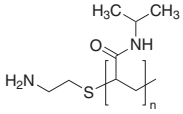
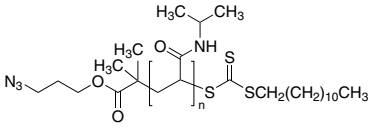
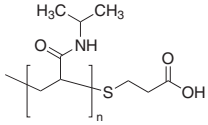
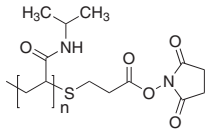
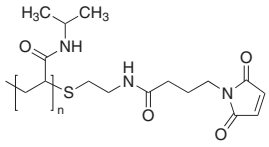
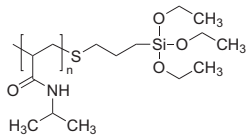
Name	Structure	Molecular Weight	Prod. No.
Poly(*N*-isopropylacrylamide)		M_n 20,000-40,000	535311-10G

End-functionalized PNIPAM

For more information on these products, visit aldrich.com/polynipam.

Name	Structure	Molecular Weight	Prod. No.
Poly(*N*-isopropyl acrylamide), amine terminated		average M_n 2000	799564-1G
		average M_n 5,000	802107-1G
Poly(*N*-isopropylacrylamide), amine terminated		M_n 2000-3000	724823-1G 724823-5G
		average M_n 5,500	724831-1G 724831-5G
Poly(*N*-isopropylacrylamide), azide terminated		average M_n 15,000	747068-1G 747068-5G
Poly(*N*-isopropylacrylamide), carboxylic acid terminated		average M_n 2,000	724815-1G 724815-5G
		average M_n 5,000	724807-1G 724807-5G
		average M_n 7,000	724866-1G 724866-5G
		average M_n 10,000	724459-5G
Poly(*N*-isopropylacrylamide), *N*-hydroxysuccinimide (NHS) ester terminated		average M_n 2,000	725668-1G 725668-5G
Poly(*N*-isopropylacrylamide), maleimide terminated		average M_n 2,000	731048-1G 731048-5G
		average M_n 5500	728632-1G 728632-5G
Poly(*N*-isopropylacrylamide) triethoxysilane terminated		average M_n 2,750	760978-1G 760978-5G

PolyNIPAM Copolymers

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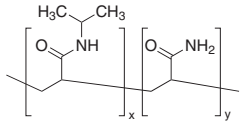
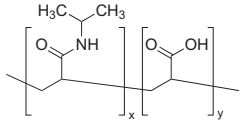
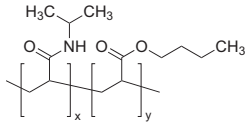
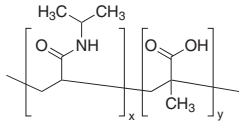
Name	Structure	Molecular Weight/Viscosity	Prod. No.
Poly(*N*-isopropylacrylamide-*co*-acrylamide)		average M_n 20,000	738727-5G
Poly(*N*-isopropylacrylamide-co-acrylic acid)		viscosity 7500-12500 5 % in H_2O	741930-5G
Poly(*N*-isopropylacrylamide-*co*-butylacrylate)		average M_n 30,000	762857-5G
Poly(*N*-isopropylacrylamide-*co*-methacrylic acid)		average M_n 8,000-10,000	750166-5G
		M_n 30,000-50,000	724467-5G

Controlled Release | Targeted Delivery | Solubility Enhancement

SHAPE CHANGE POLY(N-ISOPROPYLACRYLAMIDE) MICROSTRUCTURES FOR DRUG DELIVERY



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Introduction

Therapeutic drugs have evolved from single component formulations such as powders to multi-component drug delivery systems (DDSs) such as capsules, anisotropic particles, or microfabricated needle patches. An effective DDS must be both smart and multifunctional; it must release a drug at a specified anatomical location within the therapeutic range for a specified period of time and with minimal side effects. DDSs can target cell receptors using antibodies, ligands, or aptamers for more effective cancer therapeutics[1] or dissolve and release their cargo in specific anatomical areas such as the stomach, which is highly acidic.[2] Thus far, while polymeric DDSs can bind to specific cells or be broken apart by biochemical reactions, they are inherently static and do not reconfigure or change shape dynamically. Shape change is an emerging concept in DDSs which is inspired by robotics and the tunable shapes of cells or pathogens. Shape change offers the possibility for autonomous, environmentally responsive multi-state functionality. There are a number of dynamic polymeric DDSs that range in size from nanometer-sized biomolecular constructs to centimeter-sized implants. These new materials are in different stages of development, ranging from laboratory curiosity to clinical trial. Nanometer-sized dynamic shape change structures for use in DDSs are composed of smart biomolecules, such as DNA, and can be assembled into shapes such as cubical containers with controllable lids.[3] In this article, we restrict our discussion to larger, micro or mesoscale systems and focus on the use of shape change polymer microstructures to create dynamic DDSs based on reversible swelling.

Shape Change Polymer Microstructures

Shape memory polymers and hydrogels are perhaps the most important types of all responsive polymers.[4–6] These systems often are composed of two kinds of molecular moieties, including rigid and flexible chains or hydrophobic and hydrophilic structural units. While the use of shape memory polymers in drug delivery is reviewed elsewhere,[7] we elaborate here on multilayer and patterned microstructures composed of at least one hydrogel component. This paradigm represents an attractive concept for dynamic DDSs for the following reasons:

- Hydrogels have mechanical properties, such as moduli, that are well matched with those of human tissue and organs. Many are biocompatible and capable of swelling by several orders of magnitude in volume in response to a range of different environmental stimuli.[8,9] In aqueous biological systems, swelling or collapse can occur due to absorption or expulsion of water.
- Hydrogel swelling can be achieved in response to various stimuli like pH,[10] temperature,[11] electric field,[12] or biomolecules[13–17] and can be programmed to be responsive to more than one stimulus at a time.[18–20] In addition, gelation can also be triggered *in vivo*.[21]
- Due to large changes in volume, swelling and de-swelling cause large mechanical deformations that can be used to enable actuation without the need for any other external sources of energy such as batteries or wires.
- Multilayers[22] using combinations of more and less swelling hydrogels or gradient crosslinked hydrogels can be designed so that differential volume change during swelling can be converted to spontaneous curving and folding to form a wide range of three-dimensional (3D) shapes and structures.[23–25]
- With advances in bio-MEMS, a number of techniques have been developed to pattern and structure hydrogels in a wide range of shapes and sizes using mass-producible methods such as photolithography, printing, molding, or layer by layer assembly methods.

An Example: Theragrippers

Self-folding DDSs, referred to as theragrippers for chemomechanical controlled drug release,[26] are hand-shaped DDSs that contain multiple digits that can open and close in response to a change of temperature or pH. The shape change is actuated by Poly(N-isopropylacrylamide) (pNIPAM), a thermoresponsive hydrogel[27] that has been studied extensively for use in drug delivery and for the creation of stiffer scaffold materials for surgical applications. The swelling of pNIPAM is driven by the transition from a hydrophilic water-absorbing state to a hydrophobic water-expelling state as the temperature increases above physiological temperature. pNIPAM is traditionally formulated using photolithography, micromolding, emulsion polymerization or radical polymerization techniques.[28] Shah et al. has reviewed methods to create monodispersed pNIPAM microgels using microfluidic devices as well as manipulation of the conditions required to induce gelation.[29]

Crosslinked pNIPAM is typically a relatively soft material with a modulus of approximately 150 KPa. To increase rigidity of the theragrippers for gripping applications, the highly swelling gel is paired with a stiff non-swelling polypropylene fumarate (PPF) with a significantly higher modulus of 16 MPa to form a bilayer. In addition, NIPAM was co-polymerized with acrylic acid (AAc) to endow pH sensitivity. This bilayer was patterned using photolithography in the shape of hands in a mass-producible manner (**Figure 1**), on a sacrificial layer of polyvinyl alcohol using a protocol discussed below. Several schemes were used to load drugs such as mesalamine and doxorubicin into the theragrippers. These included: (a) soaking the fabricated theragrippers overnight in the drug solution when the drug soaked primarily into the porous pNIPAM hydrogel layer; (b) adding the drug to a microporous PPF layer which was created by leaching salt that was earlier mixed into the PPF layer; and (c) incorporating the dry drug powder into the PPF mixture before photocrosslinking, which allowed a more uniform distribution. Gripping of cells was verified *in vitro*, and drug release was verified *in vivo* in the stomach of a live pig (**Figure 2**).[26] In addition, such responsive polymer microstructures can be loaded with magnetic nanoparticles for manipulation from afar using magnetic fields, allowing for the possibility for remote guidance.[30]

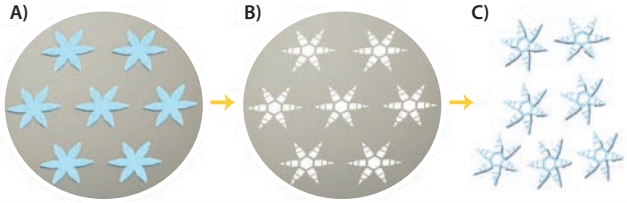

Figure 1. Microfabricated theragrippers. Mass production of hand-shaped bilayer microstructures fabricated by photopatterning, **A**) a hand-shaped stimuli-responsive pNIPAM hydrogel, and **B**) a segmented layer of a stiff polymer PPF on a substrate followed by **C**) release from the substrate.

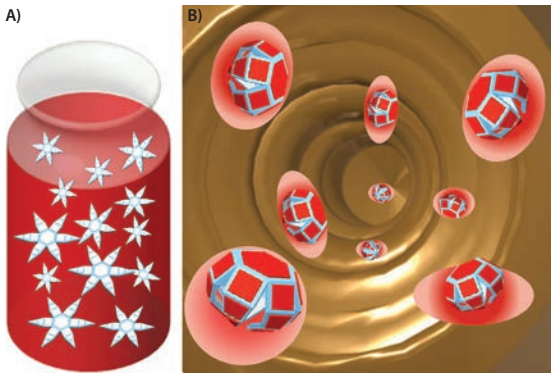

Figure 2. Loading and delivery of drugs using theragrippers. **A**) Absorption of drugs on porous hydrogel. **B**) Free-standing soft grippers for drug delivery.

Outlook and Challenges

Shape change self-folding hydrogel microstructures offer many advantages as DDSs. In the future, we envision applications such as self-attaching devices for sustained drug release, self-coiling devices for blocking aneurisms, self-expanding drug eluting stents, and self-propelled and miniaturized locomotive devices.

Since DDSs are envisioned for *in vivo* applications, stringent requirements must be met to ensure safety and minimize toxicity, which vary depending on the anatomical position and duration of drug release. In the previous theragrippers example, there is the somewhat questionable biosafety of pNIPAM in the body. On the one hand, pNIPAM is widely used in cell culture dishes where no significant cellular toxicity has been observed; also, it can be created with biodegradable crosslinkers offering the possibility for clearance from the body.[31–33] Additionally, ophthalmic formulations of pNIPAM have been reported with no *in vitro* cytotoxicity.[34] On the other hand, the NIPAM monomers are potentially toxic *in vivo*.[35] Consequently, we emphasize that this example of a pNIPAM theragripper is being used for illustrative purposes and more research needs to be done to evaluate its safety. Additional research is needed, as well, on developing such shape change microstructures using alternate bio-friendly soft-material compositions such as chitosan,[36] gelatin,[37] cellulose,[38] alginate and hyaluronic acid,[39] PLGA,[40] PVA,[41] PEO, and PEO-PPO-PEO block copolymers like Pluronic® block copolymers or poloxamers.[42,43]

Due to significant advances in nanotechnology and biofabrication, future DDSs are expected to embody more characteristics of living systems such as self-assembly and disassembly, energy dissipation and adaptation. They will be dynamic and exquisitely structured at a variety of length scales. Much like a complex macroengineered robotic device they would likely incorporate feedback, control, logic, and possibly memory that is manifested through a combination of molecular binding, chemical reaction and electronic circuits. As highlighted here, the reversible swelling of hydrogels and their engineering into curved and folded 3D microstructures, in the absence of any external power sources, could be used to enable a variety of autonomous shape changes and, consequently, smart behaviors that are a small step to enabling the grand vision of the ideal therapeutic.

Method: Fabrication of Theragrippers

This is the protocol that is used for fabricating PPF/pNIPAM grippers in the Gracias Laboratory.[26,44,45]

1. Preparation of the stock solutions
   a) *PVA stock solution:* prepare a 30% PVA (9,000 Da M_w, 80% hydrolyzed, **Aldrich Prod. No.** 360627) solution by weight in deionized water.
   b) *pNIPAM-AAc stock solution*[44]*:* NIPAM monomer 3 g, (**Aldrich Prod. No.** 415324) is mixed with pNIPAM 0.4 g, (**Aldrich Prod. No.** 535311) and Bis-acrylamide (0.18 g, **Sigma-Aldrich Prod. No.** 146072) in 7.5 mL of n-butanol (**Sigma-Aldrich Prod. No.** 34867) as a solvent. This solution is stirred overnight and the undissolved crystals are separated prior to use by decantation. Before decanting, mix 0.31 mL of acrylic acid (**Aldrich Prod. No.** 147230) into the pNIPAM and NIPAM solution.
   c) *PPF stock solution*[45]*:* it should be noted that the polymer PPF was synthesized in the laboratory of John Fisher at the University of Maryland. The synthesis for PPF summarized below is based on their published protocol.[45] Briefly, it involves reacting a 3:1 molar ratio of propylene glycol (**Aldrich Prod. No.** 309028) with diethyl fumarate (**Aldrich Prod. No.** D95654) in an inert atmosphere with incrementally increasing stir rates. 0.01 moles of $ZnCl_2$ (**Aldrich Prod. No.** 229997) (catalyst) and 0.002 moles of hydroquinone (**Sigma-Aldrich Prod. No.** H9003) (crosslinking inhibitor) are added per mole of diethyl fumarate. The intermediate, bis(hydroxypropyl) fumarate is formed upon increasing the reaction temperature gradually up to 130 °C and ethanol as a byproduct distillate. Collection of 90% of the theoretical yield of ethanol indicates the termination point of the reaction.

      Following the first reaction, under reduced pressure conditions (less than 1 torr) and a gradual increase in temperature to 130 °C, the trans-esterification of the intermediate produces the PPF polymer and the byproduct, propylene glycol. Gel permeation chromatography (GPC) is used to determine the end point of the reaction when the required molecular weight of PPF is obtained (in our case, 952 Da). A series of purification steps are done, with the polymer first being dissolved in methylene chloride and subsequently washing with hydrochloric acid (**Sigma-Aldrich Prod. No.** 320331), distilled water, and brine solution to remove the catalyst and smaller chain polymers. Sodium sulfate and ethyl ether washes, followed by evaporation under reduced pressure, are used to rid the PPF solution of the solvents.
2. **Sacrificial layer**—spin-coat the PVA solution onto a clean silicon wafer at 3,000 rpm and dry on a hot plate at 115 °C for 5 minutes.
3. **PPF layer**—spin-coat the PPF solution onto the sacrificial layer at 3,000 rpm and expose to UV light of intensity 650 mJ/cm^2 using a Quintel mask aligner.
   a) *pNIPAM-AAc layer:* add 100 µL of Irgacure 2100 (photoinitiator, Ciba) to the pNIPAM solution, keep the solution bottle covered with a foil cover throughout the remainder of the fabrication process. Spin-coat one mL of this solution onto the the PPF layer and expose to UV light of intensity 50 mJ/cm^2. Ethanol, followed by DI water, is used to develop these 2 layers and reveal the theragripper features.
   b) *Releasing the theragrippers:* soak the silicon wafer with attached theragrippers in DI water for 20 minutes to dissolve the PVA layer to release the structures.
4. Loading drugs onto the theragrippers[26]
   a) *Method 1:* Upon release, soak the theragrippers overnight in a chemical solution. The hydrophilicity and swelling capacity of the pNIPAM hydrogel layer will cause it to soak up more of the chemical solution than the PPF layer.
   b) *Method 2:* Add 5% NaCl solution to PPF, and spin-coated as in Step 3. Upon releasing the grippers using DI water, the water will also leach the salt out of the PPF layer to create porosity within the PPF layer. Soak in chemical solution to allow the drug to enter both layers of the theragrippers.
   c) *Method 3:* Dry load drug by mixing the dry drug powder into the PPF layer, and then crosslinking the polymer, to incorporate the drug into the polymer mesh of the PPF layer. Drug release is controlled both *in vitro* and *in vivo*.

References

(1) Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O. C.; Margalit, R.; Langer, R. *Nat. Nanotechnol.* **2007**, *2*, 751–760.
(2) Yoshida, T.; Lai, T. C.; Kwon, G. S.; Sako, K. *Expert Opin. Drug Deliv.* **2013**, *10*, 1497–1513.
(3) Andersen, E. S.; Dong, M.; Nielsen, M. M.; Jahn, K.; Subramani, R.; Mamdouh, W.; Golas, M. M.; Sander, B.; Stark, H.; Oliveira, C. L.; Pedersen, J. S.; Birkedal, V.; Besenbacher, F.; Gothelf, K. V.; Kjems, J. *Nature* **2009**, *459*, 73–76.
(4) Yoo, J. W.; Doshi, N.; Mitragotri, S. *Adv. Drug Deliv. Rev.* **2011**, *63*, 1247–1256.
(5) Qiu, Y.; Park, K. *Adv. Drug Deliv. Rev.* **2012**, *64*, 49-60
(6) Wischke, C.; Behl, M.; Lendlein, A. Expert Opin. Drug Deliv. **2013**, *10*, 1193–1205.
(7) Lendlein, A.; Behl, M.; Hiebl, B.; Wischke, C. *Expert Rev. Med. Devices* **2010**, *7*, 357–379.
(8) Chang, C.; Duan, B.; Cai, J.; Zhang, L. *Eur. Polym. J.* **2010**, *46*, 92–100.
(9) Okay, O. *Springer Ser Chem Se* **2010**, *6*, 1–14.
(10) Binauld, S.; Stenzel, M. H. *Chem. Commun.* **2013**, *49*, 2082–2102.
(11) Gong, C.; Qi, T.; Wei, X.; Qu, Y.; Wu, Q.; Luo, F.; Qian, Z. *Curr. Med. Chem.* **2013**, *20*, 79–94.
(12) Murdan, S. *J. Controlled Release* **2003**, *92*, 1–17.
(13) Miyata, T.; Asami, N.; Uragami, T. *Macromolecules* **1999**, *32*, 2082–2084.
(14) Miyata, T.; Asami, N.; Uragami, T. *J. Polym. Sci., Part B: Polym. Phys.* **2009**, *47*, 2144–2157.
(15) Leong, T. G.; Randall, C. L.; Benson, B. R.; Bassik, N.; Stern, G. M.; Gracias, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 703–708.
(16) Bassik, N.; Brafman, A.; Zarafshar, A. M.; Jamal, M.; Luvsanjav, D.; Selaru, F. M.; Gracias, D. H. *J. Am. Chem. Soc.* **2010**, *132*, 16314–16317.
(17) Hu, J.; Zhang, G.; Liu, S. *Chem. Soc. Rev.* **2012**, *41*, 5933–5949.
(18) Sershen, S. R.; Westcott, S. L.; Halas, N. J.; West, J. L. *J. Biomed. Mater. Res.* **2000**, *51*, 293–298.
(19) Zhang, X.; Wu, D.; Chu, C. C. *Biomaterials* **2004**, *25*, 4719–4730.
(20) Fusco, S.; Huang, H. W.; Peyer, K. E.; Peters, C.; Haberli, M.; Ulbers, A.; Spyrogianni, A.; Pellicer, E.; Sort, J.; Pratsinis, S. E.; Nelson, B. J.; Sakar, M. S.; Pane, S. *ACS Appl. Mater. Interfaces* **2015**, *7*, 6803–6811.
(21) Patenaude, M.; Hoare, T. *ACS Macro Lett.* **2012**, *1*, 409–413.
(22) Gracias, D. H. *Curr. Opin. Chem. Eng.* **2013**, *2*, 112–119.
(23) Guan, J.; He, H.; Hansford, D. J.; Lee, L. J. *J. Phys. Chem. B* **2005**, *109*, 23134–23137.
(24) Stoychev, G.; Puretskiy, N.; Ionov, L. *Soft Matter* **2011**, *7*, 3277–3279.
(25) Fernandes, R.; Gracias D. H. *Adv. Drug Del. Rev.* **2012**, *64*, 1579–1589.
(26) Malachowski, K.; Breger, J.; Kwag, H. R.; Wang, M. O.; Fisher, J. P.; Selaru, F. M.; Gracias, D. H. *Angew. Chem.* **2014**, *126*, 8183–8187.
(27) Hirokawa, Y.; Tanaka, T. *J. Chem. Phys.* **1984**, *12*, 6379–6380.
(28) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, *33*, 448–477.
(29) Shah, R. K.; Kim, J. W.; Agresti, J. J.; Weitz, D. A.; Chu, L. Y. *Soft Matter.* **2008**, *4*, 2303–2309.
(30) Breger, J. C.; Yoon, C.; Xiao, R.; Kwag, H.; Wang, M. O.; Fisher, J. P.; Nguyen, T. D.; Gracias, D. H. *ACS Appl. Mater. Interfaces.* **2015**, *7*, 3398–3405.
(31) Schild, H. G. *Prog.Polym. Sci.* **1992**, *17*, 163–249.
(32) Kavanagh, C. A.; Gorelova, T. A.; Selezneva, I. I.; Rochev, Y. A.; Dawson, K. A.; Gallagher, W. M.; Gorelov, A. V.; Keenan, A. K. *J. Biomed. Mat. Res., Part A* **2005**, *72*, 25–35.
(33) Naha, P. C.; Bhattacharya, K.; Tenuta, T.; Dawson, K. A.; Lynch, I.; Gracia, A.; Lyng, F. M.; Byrne, H. J. *Toxicol. Lett.* **2010**, *198*, 134–143.
(34) Hsiue, G. H.; Hsu, S. H.; Yang, C. C.; Lee, S. H.; Yang, I. K. *Biomaterials* **2002**, *23*, 457–462.
(35) Vihola, H.; Laukkanen, A.; Valtola, L.; Tenhu, H.; Hirvonen, J. *Biomaterials* **2005**, *26*, 3055–3064.
(36) Bhattarai, N.; Gunn, J.; Zhang, M. *Adv. Drug Delivery Rev.* **2010**, *62*, 83–99.
(37) Kurisawa, M.; Yui, N. *J. Controlled Release* **1998**, *54*, 191–200.
(38) Kulkarni, R. V.; Sa, B. *Drug Dev. Ind. Pharm.* **2008**, *34*, 1406–1414.
(39) Matricardi, P.; Di Meo, C.; Coviello, T.; Hennink, W. E.; Alhaique, F. *Adv. Drug Del. Rev.* **2013**, *65*, 1172–1187.
(40) Kim, D. H.; Martin, D. C. *Biomaterials* **2006**, *27*, 3031–3037.
(41) Perennes, F.; Marmiroli, B.; Matteucci, M.; Tormen, M.; Vaccari, L.; Di Fabrizio, E. *J. Micromech. Microeng.* **2006**, *16*, 473–479.
(42) Pillai, O.; Panchagnula, R. *Curr. Opin. Chem. Biol.* **2001**, *5*, 447–451.
(43) He, C.; Kim; S. W.; Lee, D. S. *J. Controlled Release* **2008**, *127*, 189–207.
(44) Bassik, N.; Abebe, B. T.; Laflin, K. E.; Gracias, D. H. *Polymer* **2010**, *51*, 6093–6098.
(45) Kasper, F. K.; Tanahashi, K.; Fisher, J. P.; Mikos, A. G. *Nat. Protoc.* **2009**, *4*, 518–525.

Controlled Release | Targeted Delivery | Solubility Enhancement

FORMULATION OF POLY(ETHYLENE GLYCOL) HYDROGELS FOR DRUG DELIVERY


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Introduction

Hydrogels are an attractive vehicle for localized administration of pharmaceutical agents such as proteins and small molecules that can be released in a temporally and spatially controlled manner. By maintaining a high local concentration of a drug, hydrogels bypass the need for systemic drug administration and provide a reservoir of the drug to be released slowly over time. Poly(ethylene glycol) (PEG) is a hydrophilic polymer that, when crosslinked and swollen with water, produces a three-dimensional (3D) hydrogel capable of encapsulating drugs and cells. Therefore, PEG hydrogels have been studied extensively as a platform for drug delivery and tissue engineering. PEG hydrogels may be an ideal drug delivery vehicle due to favorable host-material interactions: they are non-toxic, biocompatible, resist biofouling, and are non-biodegradable.[1] The mechanical properties of PEG hydrogels are also well-defined and can be tuned for application, including the drug delivery site and drug diffusion kinetics, by modifying PEG molecular weight and crosslink density.[2] Functionalization of PEG allows for further robust modification of the chemical and mechanical properties of PEG hydrogels. But perhaps the simplest synthetic addition is acrylation, which allows crosslinking via free radical additions (**Figure 1**).

PEG can be end-functionalized with a variety of other groups, including methoxy, hydroxy, maleimide, thiol, and azide moieties, all of which are commercially available in homobifunctional and heterobifunctional forms. The diversity of functional groups allows unique crosslinkers with different crosslinking chemistry, such as Michael-type additions, thiol-ene and Diels-Alder click reactions, enzymatic reactions, and carbonyl additions.[3] These reactions can be triggered with ultraviolet light, changes in pH, temperature, electromagnetic fields, or the addition of other chemical compounds.[4]

PEG-hydrogel Drug Delivery Applications

Drug delivery from a hydrogel involves the diffusion of an encapsulated pharmaceutical agent through the bulk of the gel into the immediate microenvironment surrounding the delivery site. Depending on the porosity, hydrophilicity, and other physicochemical properties of the hydrogel and the drug, the loaded drug can elute slowly over time in a pharmacokinetically controlled manner that prolongs circulation time.[5] Many different agents have been used in PEG-containing hydrogel drug delivery applications, including small molecules,[6] macromolecules,[7] and nano/microparticles.[8–9] Such formulations have been applied to cutaneous, ocular, and cardiac tissue, among others.

Although described as a "bioinert" drug delivery platform, PEG hydrogels can also incorporate bioactive materials, such as extracellular matrix amino acid motifs or macromolecules such as collagen.[10] Incorporation of biological macromolecules facilitates integration into the native tissue environment due to cell-integrin recognition of extracellular matrix components such as laminin, fibronectin, collagen, and hyaluronic acid. This may be important for drug delivery applications where degradation of the hydrogel, and therefore drug release, should be mediated by the host microenvironment. Control of degradation is, therefore, critically dependent on the composition of the hydrogel and the sensitivity of the degradable sequences.

Interpenetrating Networks of PEG and Biological Materials

Due to the poor mechanical stability of biologically derived hydrogels such as collagen, many have incorporated PEG into the polymer matrix to enhance the mechanical strength and longevity *in vivo*. In general, interpenetrating networks (IPNs) consist of a binary system of two polymers that are chosen to better control the physical and biological properties of the hydrogel. Fusion of synthetic and biological materials can be via physical entanglement or covalent crosslinking. Several groups have incorporated hyaluronic acid into degradable PEG hydrogels in an IPN to increase cell proliferation and activity for cartilage, cutaneous, vocal folds and other soft tissue.[11–12] The PEG in these formulations is typically modified with poly(lactic acid) or similar copolymer to facilitate degradation. Another formulation uses chitosan, a natural polysaccharide derived from crustacean shells, often used in wound healing applications.[13]

A robust and versatile IPN platform that consists of cysteine-conjugated gelatin processed from collagen (Gel-PEG-Cys), and PEG-diacrylates

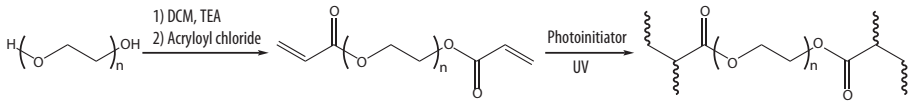


Figure 1. Synthesis of a PEG-diacrylate hydrogel.

(PEGdA) has been developed (**Figure 2**).[14–15] Both components of the IPN, gelatin and PEGdA, are crosslinked together via a thiol-ene reaction with or without UV light *in situ*. In comparison to a physically entangled network of gelatin and crosslinked PEGdA, the IPN shows improved mechanical properties: depending on the ratio of gelatin to PEG, the elastic modulus can range from 1 to 360 MPa, whereas fixed gelatin alone is ~0.1 MPa.[16] *In vivo* degradation can be further modified by adjusting a number of parameters, including the gelatin: PEGdA ratio, gelatin strength bloom, concentration and percent thiol modification, PEGdA concentration and molecular weight, and initiator weight percent.[17] This IPN biomatrix has been used to encapsulate various cell types such as fibroblasts, keratinocytes, and mesenchymal stromal/stem cells as well as small molecular weight drugs such as minocycline, silver sulfadiazine, bupivicaine, and biologics such as peptides and growth factors.[14,18–21] Further, this IPN can be used as a platform that can be paired with other drugs or drug combinations for site-specific delivery.

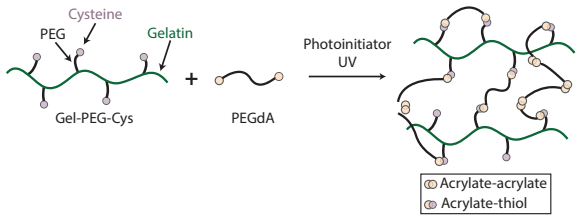


Figure 2. Interpenetrating network composed of cysteine-conjugated gelatin and PEG-diacrylate.

Method: Formulation of PEG-gelatin IPN Hydrogel Using UV Polymerization*

The following procedure creates 5 mL of IPN hydrogel containing poly(ethylene glycol) diacrylate and cysteine-conjugated gelatin. If additional components are added to the IPN, the solubility of those components should be determined at the appropriate concentration prior to incorporation into the IPN. A wide range of bioactive compounds was tested and verified using this formulation, as listed in **Table 1**. The viability of cells, if incorporated into the hydrogel, should also be assessed. Hydrogel composition can be varied by mixing different volumes of the PEG-diacrylate (PEGdA) and modified gelatin-Cys (Gel-PEG-Cys) solutions (Step 5).

1. Add 5.0 mL H_2O to the vial containing 5 mg of photoinitiator (**Aldrich Prod. No.** 410896) to create a 0.1% photoinitiator solution.
2. Warm the photoinitiator solution to 60 °C and vortex occasionally until dissolved; then allow the photoinitiator solution to return to 37 °C.
3. Add optional soluble factors (growth factors, small molecules, other macromolecules) at desired concentration to photoinitiator solution (see **Table 1** for a list of materials).
4. Allow the PEGdA (**Aldrich Prod. No.** 806757*) and Gel-PEG-Cys (**Aldrich Prod No.** 806730*) vials to reach room temperature.
5. Pipette 2.5 mL of the photoinitiator solution into the Gel-PEG-Cys vial and 2.5 mL of the photoinitiator solution into the PEGdA vial. Warm solutions to 37 °C and mix gently by pipetting up and down.
6. Add the PEGdA solution to the Gel-PEG-Cys solution and mix gently by pipetting up and down.
7. Pipette the hydrogel solution into desired mold or location.
8. Polymerize hydrogel for about 3 minutes under a long-wavelength UV lamp (CF1,000 LED, λ_{max} = 365 nm; Clearstone Technologies, Minneapolis, MN or equivalent).

*Note: PEGdA and Gel-PEG-Cys are available as kit components of **Aldrich Prod. No.** 799610.

Method: Formulation of PEG-gelatin IPN Hydrogel Without UV Polymerization*

This formulation does not require a photoinitiator or UV exposure to crosslink the network and is ideal for loading bioactives that are known to be UV-sensitive. Crosslinking proceeds via a Michael-type addition between the thiol and acrylate moieties upon a shift to a basic pH.[22]

1. Add optional soluble factors (growth factors, small molecules, other macromolecules) at desired concentration to sterile water.
2. Warm the water solution to 37 °C.
3. Allow the PEGdA (**Aldrich Prod. No.** 806757*) and Gel-PEG-Thiol (**Aldrich Prod. No.** 806749*) vials to reach room temperature.
4. Pipette 2.5 mL of the water solution into the Gel-PEG-Thiol vial and 2.5 mL of the water solution into the PEGdA vial. Warm solutions to 37 °C. Mix gently by pipetting up and down.
5. Add the PEGdA solution to the Gel-PEG-Thiol solution and mix gently by pipetting up and down.
6. Titrate to pH 8.5 with 1 N NaOH.
7. Quickly pipette the hydrogel solution into desired mold or location.
8. The PEGdA/Gel-PEG-Thiol gelatin hydrogel will polymerize within 3 min.

*Note: PEGdA and Gel-PEG-Thiol are available as kit components of **Aldrich Prod. No.** 799629.

Table 1. Materials

Item	Prod. No.
PHSRN (fibronectin domain)	161044K
Optional Bioactives	
Dexamethasone sodium phosphate	D0720000
Chlorhexidine	282227
Parvalbumin	P6393
Basic human fibroblast growth factor	F0291
Methylprednisolone acetate	M1755000
Keratinocyte growth factor	K1757
Silver sulfadiazine	481181
Bupivacaine hydrochloride	1078507
Sulfadiazine sodium	S6387
Bovine serum albumin	05470

References

(1) Hoffman, A. S. *Adv Drug Deliv Rev* **2002**, *54* (1), 3–12.
(2) Browning, M. B.; Wilems, T.; Hahn, M.; Cosgriff-Hernandez, E. *J Biomed Mater Res A* **2011**, *98* (2), 268–273.
(3) Hennink, W. E.; van Nostrum, C. F. *Adv Drug Deliv Rev* **2002**, *54* (1), 13–36.
(4) Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. *Eur J Pharm Biopharm* **2000**, *50* (1), 27–46.
(5) Hoare, T. R.; Kohane, D. S. *Polymer* **2008**, *49* (8), 1993–2007.
(6) Peppas, N. A.; Keys, K. B.; Torres-Lugo, M.; Lowman, A. M. *J. Controlled Release* **1999**, *62* (1-2), 81–87.
(7) Saito, N.; Okada, T.; Horiuchi, H.; Murakami, N.; Takahashi, J.; Nawata, M.; Ota, H.; Nozaki, K.; Takaoka, K. *Nature Biotechnology* **2001**, *19* (4), 332–335.
(8) Hamidi, M.; Azadi, A.; Rafiei, P. *Adv Drug Deliv Rev* **2008**, *60* (15), 1638–1649.
(9) Holland, T. A.; Tabata, Y.; Mikos, A. G., *J. Controlled Release* **2003**, *91* (3), 299–313.
(10) Zhu, J. M., *Biomaterials* **2010**, *31* (17), 4639–4656.
(11) Kutty, J. K.; Cho, E.; Soo Lee, J.; Vyavahare, N. R.; Webb, K. *Biomaterials* **2007**, *28* (33), 4928–4938.
(12) Skaalure, S. C.; Dimson, S. O.; Pennington, A. M.; Bryant, S. J. *Acta Biomater* **2014**, *10* (8), 3409–3420.
(13) Lee, S. J.; Kim, S. S.; Lee, Y. M. *Carbohydrate Polymers* **2000**, *41* (2), 197–205.
(14) Fu, Y.; Xu, K.; Zheng, X.; Giacomin, A. J.; Mix, A. W.; Kao, W. J. *Biomaterials* **2012**, *33* (1), 48–58.
(15) Xu, K.; Fu, Y.; Chung, W.; Zheng, X.; Cui, Y.; Hsu, I. C.; Kao, W. J. *Acta Biomater* **2012**, *8* (7), 2504–2516.
(16) Burmania, J. A.; Martinez-Diaz, G. J.; Kao, W. J. *J Biomed Mater Res A* **2003**, *67* (1), 224–234.
(17) Martinez-Diaz, G. J.; Nelson, D.; Crone, W. C.; Kao, W. Y. J. *Macromolecular Chemistry and Physics* **2003**, *204* (15), 1898–1908.
(18) Guerra, A. D.; Cantu, D. A.; Vecchi, J. T.; Rose, W. E.; Hematti, P.; Kao, W. J. *AAPS J* **2015**.
(19) Kleinbeck, K. R.; Bader, R. A.; Kao, W. J. *J Burn Care Res* **2009**, *30* (1), 98–104.
(20) Cantu, D. A.; Hematti, P.; Kao, W. J. *Stem Cells Transl Med* **2012**, *1* (10), 740–749.
(21) Waldeck, H.; Chung, A. S.; Kao, W. J. *J Biomed Mater Res A* **2007**, *82* (4), 861–871.
(22) Xu, K.; Cantu, D. A.; Fu, Y.; Kim, J.; Zheng, X.; Hematti, P.; Kao, W. J. *Acta Biomater* **2013**, *9* (11), 8802–8814.

PRODUCT HIGHLIGHT

Localize Your Drug Delivery

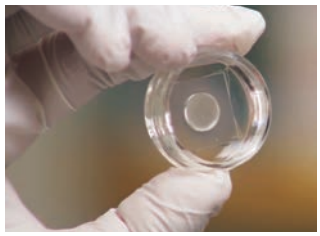
Aldrich® Materials Science offers two new ready-to-use hydrogel kits for the encapsulation and delivery of drugs, biomolecules, and cells. The hydrogels fuse the durability of synthetic polymers and the biorecognition of a natural protein to create a biocompatible delivery platform. The biodegradable matrix allows for temporally and spatially controlled delivery.

APPLICATIONS

- Localized delivery of small molecules, proteins, nucleic acids, and cellular-based therapeutics
- Tissue engineering
- Regenerative medicine

KEY BENEFITS

- Allows tailored delivery profile
- Amenable to delivery drugs and cells in a single system
- Photo or chemical crosslinking



Description	Prod. No.
Photo-crosslinkable sIPN hydrogel kit	799610-1KT
Chemically crosslinkable sIPN hydrogel kit	799629-1KT

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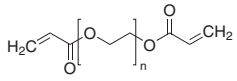
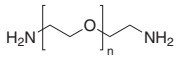
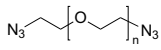
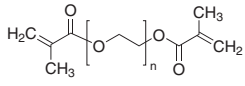
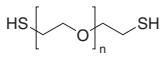
α- and ω-ends	Structure	Molecular Weight	Prod. No.
Acrylate		average M_n 2,000	701971-1G
		average M_n 6,000	701963-1G
		average M_n 10,000	729094-1G
		average M_n 20,000	767549-1G
Amine		average M_n 2,000	753084-1G 753084-5G
		average M_n 6,000	752444-1G 752444-5G
		average M_n 10,000	752460-1G 752460-5G
Azide		average M_n 10,000	767530-1G
		average M_n 20,000	756601-1G
Methacrylate		average M_n 6,000	687537-1G
		average M_n 10,000	725684-1G
		average M_n 20,000	725692-1G
SH		average M_n 1,000	717142-1G
		average M_n 3,400	704539-1G
		average M_n 8,000	705004-1G

Multi-arm PEGs

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Name	Molecular Weight	Prod. No.
Poly(ethylene oxide), 4-arm, amine terminated	average M_n 10,000	565733-250MG 565733-1G
Poly(ethylene oxide), 4-arm, carboxylic acid terminated	average M_n 10,000	565717-500MG 565717-1G
Poly(ethylene oxide), 4-arm, hydroxy terminated	average M_n 10,000	565709-1G 565709-5G
Poly(ethylene oxide), 4-arm, succinimidyl glutarate terminated	average M_n 10,000	565768-250MG 565768-1G
Poly(ethylene oxide), 4-arm, succinimidyl succinate terminated	average M_n 10,000	565741-250MG 565741-1G
Poly(ethylene oxide), 4-arm, thiol terminated	average M_n 10,000	565725-1G
Poly(ethylene oxide), 6-arm, hydroxy terminated	average M_n 17,000	570273-250MG 570273-1G

Multi-arm Block Copolymers

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Name	Molecular Weight	Prod. No.
Poly(ethylene oxide)-*block*-polylactide, 4-arm	poly(ethylene oxide) M_n 2,500 polylactide average M_n 3,500	570354-250MG 570354-1G
Poly(ethylene oxide)-*block*-polycaprolactone, 4-arm	PCL average M_n 2,500 PEG average M_n 2,500 average M_n 5,000 (total)	570346-1G

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- Drug delivery
- *In vitro* diagnostics

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Name	Prod. No.
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Type I Bovine Collagen Solution, Pepsin soluble 6 mg/mL, ≥95% purity	804622
Type I Bovine Collagen Solution, Acid Soluble telocollagen 6 mg/mL, ≥95% purity	804614

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PROTEIN PEGYLATION

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Introduction

Therapeutic proteins and other biopharmaceuticals, such as peptides and oligonucleotides, are often potent drugs that comprise an established and fast growing segment of the pharmaceuticals market. Unfortunately, proteins are prone to aggregation and misfolding, making them difficult to formulate and use. In addition, most proteins are cleared rapidly from the bloodstream upon administration. Although therapeutic protein metabolism and pharmacokinetics (PK) are complex,[1] rapid clearance can result in dose dumping, the lack of a therapeutic dose between each administration and the need for higher cumulative and more frequent dosing. Frequent dosing can result in increased occurrence of side-effects, increased risk for immunogenicity, and suboptimal efficacy. Often there is the need to extend the half-life of biopharmaceuticals in the blood to maximize clinical safety and efficacy.

Many strategies[2] have been developed and studied to optimize the pharmacokinetic properties of proteins, including hyperglycosylation,[3] fusion to an antibody Fc or to albumin, non-covalent association to albumin, and encapsulation or association with particulates.[1a,1b,4] PEGylation has been the most clinically successful strategy and involves the covalent conjugation of poly(ethylene glycol) (PEG) to the biopharmaceutical of interest (**Figure 1**). Many different proteins, peptides, and oligonucleotides have been PEGylated for use in a wide range of medical indications.[5]

Protein PEGylation was first described by Frank Davis et al. in 1977.[6] The first therapeutic PEGylated products (PEG-enzymes) appeared in the early 1990s and PEGylated cytokines were registered for clinical use by the early 2000s. The field developed rapidly thereafter. Currently, there are at least 12 innovative PEGylated biopharmaceuticals registered for clinical use, with many more in development. The most recent of these is Plegridy® (2014).[7] A number of PEGylated products have been developed from essentially the same parent protein, and several PEGylated proteins including PEGylated interferon-a2 (Pegasys® and PegIntron®) and PEGylated GCSF (Neulasta® and Lonquex™) have been developed as first-line treatments. PEGylation is also now a viable strategy for the life-cycle management of unmodified proteins in order to develop improved or biobetter versions of existing therapeutics. With the expiration of patents on the first PEGylated products about to occur, biosimilar versions are rapidly being developed, along with the required development of international standards[8] to govern this new type of generic therapeutic. The PEGylation of many other molecules (peptides, oligonucleotides, and low molecular-weight chemical entities) is also an active area of research.[5b,9]

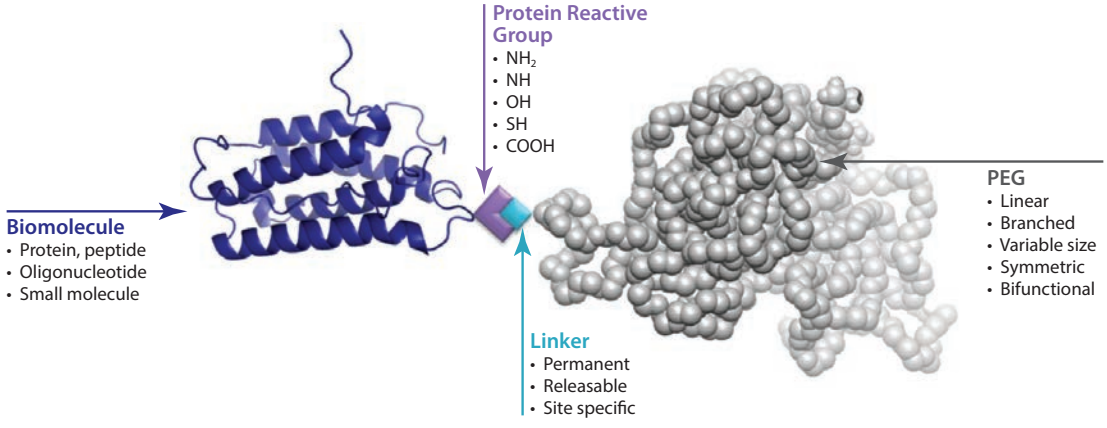

Figure 1. Schematic of a PEGylated biopharmaceutical. (Image courtesy of Dr. Karolina Peciak.)

Protein Modification

Comprehensive reviews have been published describing the broad spectrum of conjugation reactions used for protein PEGylation[5b,10] and conjugation in general. PEGylation is also used for a wide range of other applications such as PEGylating the surface of particulates.[11] Recent advances in PEGylation conjugation also are being adapted to make complex, multi-functional therapeutic proteins like antibody drug conjugates (ADCs).[12]

The PEGylation of a protein extends its retention in the body because the hydrophilic PEG polymer increases the hydrodynamic size of the protein in solution, slowing renal clearance of the conjugate. Moreover, PEG sterically shields the protein to decrease proteolytic degradation, opsonization, and uptake by the mononuclear phagocyte system. PEG steric shielding can also help reduce immunogenicity by shielding potential antigenic sites on the protein[5a,13] and minimize the propensity for aggregation.[14] Depending on the site of conjugation, PEG steric shielding can slow the rate of association of the modified protein with its target,[15] thereby decreasing potency. Disassociation rates for the PEGylated protein are thought to remain the same as the unmodified protein.[15] Hence, once a PEGylated protein is bound to its target, its properties are essentially the same as the unmodified protein.[16] Reduced association due to PEG shielding means *in vitro* evaluation of PEGylated proteins and macromolecular drugs generally do not correlate with *in vivo* efficacy.[17] While a log reduction in protein *in vitro* activity after PEGylation can be observed, most therapeutic proteins are very potent and remain so after PEGylation, thus achieving clinical benefit which is better than the unmodified protein.

In spite of the widespread clinical use of PEGylated proteins, there is still debate about the clinical effects of PEG accumulation and immunotoxicity.[18] In many cases, this can be attributed to other factors. For example, animal-based accumulation studies often use much higher bolus and cumulative doses than that given to humans.[19] Most therapeutic proteins[20] display immunogenic effects in some proportion of patients in the clinic, so the metabolism, toxicity, and clearance of PEGylated products can be dependent on the protein.[13b,13c] If the unmodified protein is safe, then the PEGylated variant is expected to be safe.[21] Products conjugated with many PEG molecules (liposomes, biomaterials, non-human enzymes) may raise additional questions regarding the impact of PEGylation on clinical efficacy and potential immune response. For this reason, it has become increasingly important to develop standardized assays for anti-PEG antibodies to better monitor PEG-specific immune responses.[13d,18a,22]

While PEG remains useful for protein modification, alternative natural and synthetic polymers[23] are also being examined (e.g., polyoxazolines,[24] phosphorylcholine methacrylates,[25] heparosan polymer,[26] hydroxyethyl starch[27]). Like PEG, these polymers will need to be thoroughly evaluated from a regulatory perspective, and the polymeric conjugation reagents need to be made reproducibly at scale with narrow polydispersity and defined chemical functionality.

PEGylation Reagents

Figure 2 shows various linear PEGylation reagents that are typically functionalized at one terminus (monofunctional) for conjugation to a protein (**Figure 2-1**). PEGylation reagents have been described using both established and recently developed conjugation functional groups[10c] to undergo reaction with many different amino acid residues on proteins,[5b,10a,10b] including non-native amino acid side-chains containing aldehyde and alkyne groups.[10d–f] Of the many PEGylation reagents described, those designed to undergo reductive amination with the amine of the *N*-terminal amino acid (PEG-aldehyde **Figure 2-2**), acylation of the lysine side-chain amine (PEG-*N*-hydroxysuccinimide (PEG-NHS) **Figure 2-3**), and Michael addition of the cysteine thiol (PEG-maleimide **Figure 2-4**; PEG-bis-sulfone **Figure 2-5**) are most common. Linear PEGylation reagents are generally in the range of 20–30 kDa because the synthesis of narrow molecular weight distribution methoxy-PEGs is more feasible than at higher molecular weights. This has led to the development of "branched" PEGylation reagents (**Figure 2-6** for example)[5b,10a,10b] and other reagents with a wide spectrum of PEG morphologies and configurations (e.g., polyPEG).[28]

Narrowly defined reaction conditions with different variants of PEG-aldehyde (**Figure 3-2**) used in stoichiometric excess sometimes can be found to undergo reaction predominantly with the *N*-terminal amine on a protein to give an intermediate imine (**Figure 3-7**). A borohydride reagent (e.g., $NaBH_4$, $NaCNBH_3$, or $Na(AcO)_3BH$) then needs be used to reduce the imine to give the PEGylated protein (**Figure 3-8**). Known

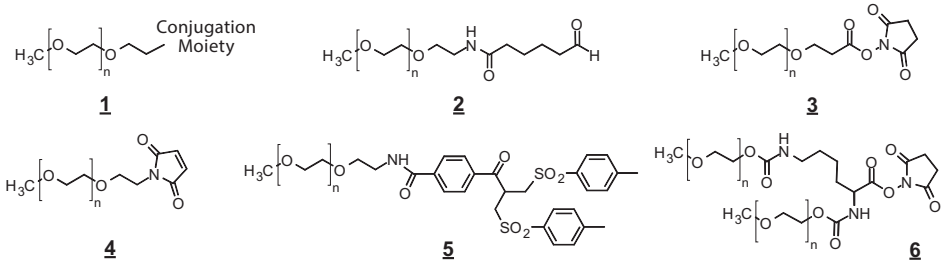

Figure 2. Linear PEGylation reagents 1 general functionalization for protein conjugation, 2 PEG-aldehyde, 3 PEG-N-hydroxysuccinimide, 4 PEG-maleimide, 5 PEG-bis-sulfone, and 6 branched PEG-*N*-hydroxysuccinimide PEGylation reagent.

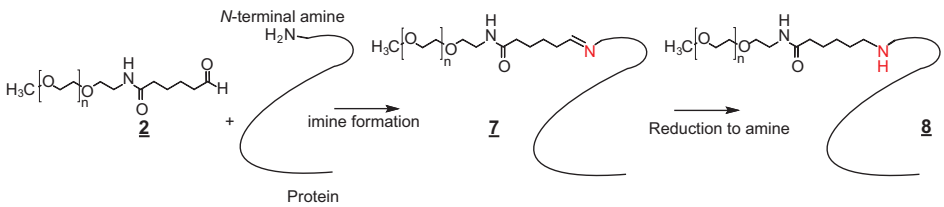

Figure 3. *N*-terminal PEGylation by reductive amination with 2 PEG-aldehyde results in an 7 intermediate imine, that can be reduced by borohydride to give the 8 *N*-terminal PEGylated protein.

as reductive amination, this strategy for PEGylation can achieve varying degrees of site-specificity at the amine of the *N*-terminal amino acid residue. Although the *N*-terminal amine can be targeted by reductive amination using a PEG-aldehyde reagent (**Figure 3-2**), specific conditions are narrow and conjugation efficiency can be low. Other strategies designed to be more efficient and selective for the *N*-terminus (or *C*-terminus) of proteins have been described.[29]

Amine PEGylation with PEG-NHS esters (**Figure 2-3**) primarily undergo non-selective acylation with lysine amines, the most accessible nucleophilic groups on the protein. However, additional amines and nucleophilic residues (terminal amine, tyrosine, serine) may also react, leading to non-specific PEGylation. Reaction of PEG-NHS with the histidine imidazole side-chain results in the formation of a hydrolytically labile carbonyl imidazolide. PEG-NHS esters are also susceptible to competitive hydrolysis which requires this reagent to be used in considerable excess.

Amine reactive reagents generally tend to be used in considerable stoichiometric excess to the target protein. Since each protein has several available nucleophilic groups, non-selective PEG conjugation results in PEG-protein positional isomers and multiple PEGylated products. Achieving both good purification and high yield can be challenging when excess PEGylation reagent is used to overcome non-selective conjugation.[30] The cost of the PEG reagent can exceed that of the unmodified protein, making conjugation efficiency during clinical development important. Conjugation of many PEG molecules, or hyper-PEGylation, has been shown to be useful for therapeutic enzymes of non-human origin that are selective for a low molecular weight substrate (e.g., Adagen®, Oncaspar®, and Krystexxa®). Hyper-PEGylation is readily achieved using amine acylating reagents such as PEG-NHS esters. Often these PEGylated products are described as being immunogenic, even though the non-PEGylated version would have no clinical utility. Non-selective mono-PEGylation with amine reactive reagents (like PEG-aldehyde **Figure 2-2** or PEG-NHS **Figure 2-3**) have been used to develop several very successful clinical products that bind to cell-surface receptors (e.g., Pegasys®, PegIntron®, Micera™, and Neulasta®).

Thiol-selective Reagents

To address cost and regulatory issues, current research efforts are underway to ensure PEGylation is more robust, efficient, and site-specific. Many site-specific approaches have been described,[10c,10d,10f] but only thiol alkylation at an α,β-unsaturated carbonyl (Michael addition reaction) will be described here. Alkylation at an α,β-unsaturated carbonyl can be rapid in mild conditions that are conducive to maintaining the tertiary structure of a protein and where protein amine nucleophiles tend to be protonated and not reactive (typically at pH 6–7). The free cysteine thiol is rare in a protein and often not available for conjugation because in the majority of proteins, cysteines are paired to form disulfides.

For thiol conjugation, PEG-maleimide (**Figure 4A-4**) has been widely used, but maleimide-derived PEG-conjugates (**Figure 4A-9**) are susceptible to deconjugation via a retro-Michael reaction and undergo thiol exchange reactions.[31] Efforts to stabilize maleimide conjugates have focused on hydrolysis of the maleimide ring after conjugation.[32] Alternative thiol-specific reagents have also been described.[33] One example is the PEG-mono-sulfone (**Figure 4B-10**)[34] which is equally reactive with PEG-maleimide. This reagent (**Figure 4B-10**) undergoes elimination *in situ* to give the reactive enone (**Figure 4B-11**) that undergoes conjugation. The initially formed PEG conjugate (**Figure 4B-13**) can be readily treated with a borohydride reagent ($NaBH_4$, $NaCNBH_3$, or $Na(AcO)_3BH$) to give a conjugate (**Figure 4B-10**) that is not susceptible to retro-Michael and other exchange reactions.

The scarcity of free cysteine thiols in therapeutic proteins has resulted in many attempts to recombinantly add a free cysteine to a protein as a site for conjugation. This strategy is viable for proteins without disulfides;[35] but for proteins with disulfides, the presence of an unpaired cysteine often results in disulfide scrambling, protein misfolding, and aggregation. Since many therapeutic proteins have disulfides, another way to exploit the selectivity and efficiency of thiol conjugation is by bis-alkylation to re-bridge disulfides using reagents such as PEG-bis-sulfone (**Figure 5-5**).[36] Elimination of the sulfinic acid leaving group

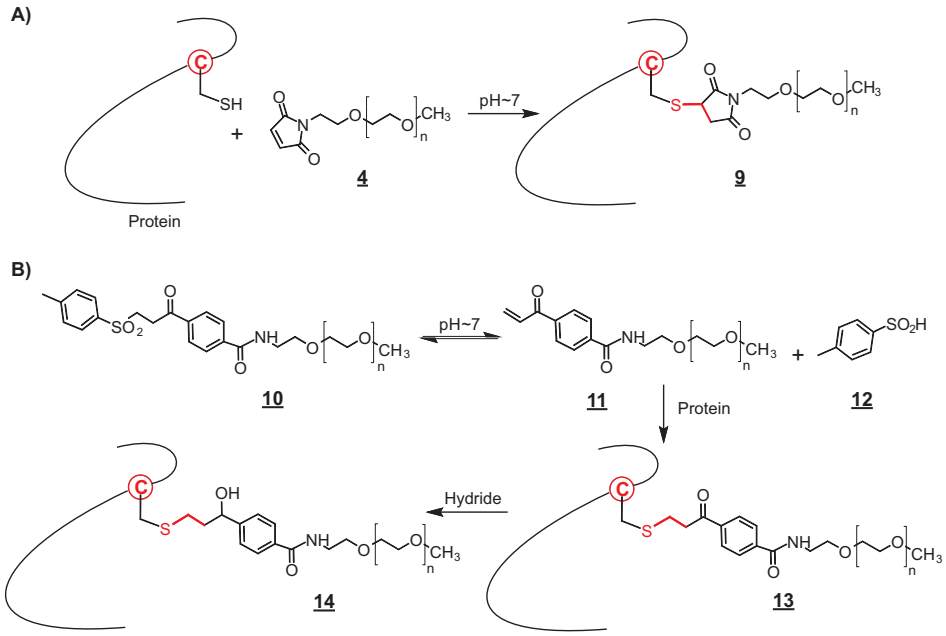

Figure 4. **A4**) PEG-maleimide, **A9**) maleimide-derived PEG-conjugate, **B10**) PEG-mono-sulfone, **B11**) elimination *in situ* to give the reactive enone, **B12**) sulfinic acid leaving group, **B13**) PEG conjugate, **B14**) stable PEG conjugated, treated with borohydride

(**Figure 5-12**) to give the PEG-mono-sulfone enone (**Figure 5-15**) allows the PEGylation reaction to proceed by what is thought to be an addition-elimination mechanism. Disulfide bridging PEGylation can be accomplished during protein refolding or after mild partial reduction of a disulfide in the purified protein. Disulfide reduction can also be readily accomplished *in situ*[37] without the need to remove the reducing agent prior to PEGylation. Although not necessary, the conjugate (**Figure 5-16**) then can be reduced without any loss of protein activity using a borohydride reagent ($NaBH_4$, $NaCNBH_3$, or $Na(AcO)_3BH$). In practice, this is not required as the hydrophobic interactions of the protein and the flexibility of the 3-carbon bridge prevent de-conjugation. Additionally, two thiols can be conjugated using a bis-alkylating maleimide linker,[38] which results in a rigid vinylic 2-carbon bridge between the thiol cysteines. As with other maleimides, hydrolysis is required to prevent de-conjugation and exchange reactions.[39]

The bis-sulfone PEGylation reagents (**Figure 5-5**) are also selective for the histidine tag (His-Tag) in proteins,[29b] which can be placed at either terminus of the protein. His-Tag PEGylation can be accomplished with greater efficiency than PEGylation by amine acylation and reductive amination.[29b] The difference in reactivity of cysteine thiols and histidine imidazoles for the PEG-bis-sulfone reagents (**Figure 5-5**) means it is possible to conduct a disulfide bridging conjugation in the presence of a His-Tag. Then this can be followed by subsequent site-specific modification at the His-Tag using the same conjugation moiety.

Summary

Drugs that utilize the properties of macromolecules can provide significant clinical benefit.[40] Protein PEGylation is a clinically proven approach where first the protein is made recombinantly, then modified by a conjugation reaction using a PEGylation reagent. Protein engineering and PEGylation are being utilized to modify[10d-f,29b] existing proteins and design non-endogeneous proteins like scaffolds[35,41] to achieve more efficient PEGylation site-selectivity. These efforts are focused to ensure there will be greater structural homogeneity, activity, and stability[42] in the final PEGylated protein product. The combination of protein engineering and chemical synthesis strategies provides a huge range of opportunities for the continued modification of proteins with PEG and other molecules.

Methods: Protein PEGylation

PEGylation of a Protein With a Single Cysteine[34]

1. The protein first can be treated with dithiothreitol (DTT) (**Sigma Prod. No.** 43815) to reduce any intermolecular disulfides that may be present. Other disulfide exchange reagents (e.g., mercaptoethylamine) can be used as well as phosphine derived reagents, e.g., tris(2-carboxyethyl)phosphine) (TCEP) (**Sigma Prod. No.** 68957). A lyophilized protein or antibody must first be resuspended in a reaction buffer such as 50 mM sodium phosphate, pH 7.4, containing 150 mM NaCl and 10 mM EDTA (0.5 mL). It is best to use ultrapure water (18.2 MΩcm) to make solutions used for PEGylation. DTT (1.0 mM stock solution) is added to the resulting solution to give a final DTT concentration of 20 mM. The resulting solution is mixed gently and then allowed to incubate at room temperature for 1 h.
2. The DTT must be removed by buffer exchange prior to the PEGylation reaction. The buffer of the reduced protein is exchanged with fresh reaction buffer using an Illustra NAP-5 column. The reduced protein concentration can be determined by measuring UV absorbance at 280 nm and the concentration should be adjusted to 0.1 mg/mL.
3. The PEGylation reagent (either PEG-maleimide **Figure 4-A4** (**Aldrich Prod. No.** 712469), or PEG-mono-sulfone **Figure 4-B10**; 1 equiv., 1 mg/mL) is added to the protein solution. The PEGylation reaction solutions are mixed gently and incubated at ambient temperature (18–25 °C) for approximately 4 h. Reaction mixtures can be analyzed by SDS-PAGE (Instant*Blue*™ stain), and then the conjugate is purified by ion-exchange chromatography.

Disulfide Bridging PEGylation[15b,37,43]

The procedure to PEGylate the two cysteine thiols[43] on a protein with PEG-bis-sulfone **Figure 5-5** is essentially the same as the PEGylation of a protein that has a single cysteine thiol available for conjugation. The key difference is the disulfide in the protein to be conjugated must first be reduced, typically using DTT or TCEP. It is often possible to use TCEP in stoichiometric equivalence to the protein to avoid the need for buffer exchange to remove the reducing agent prior to the addition of the PEGylation reagent.[37] It is also possible to use immobilized DTT or TCEP.

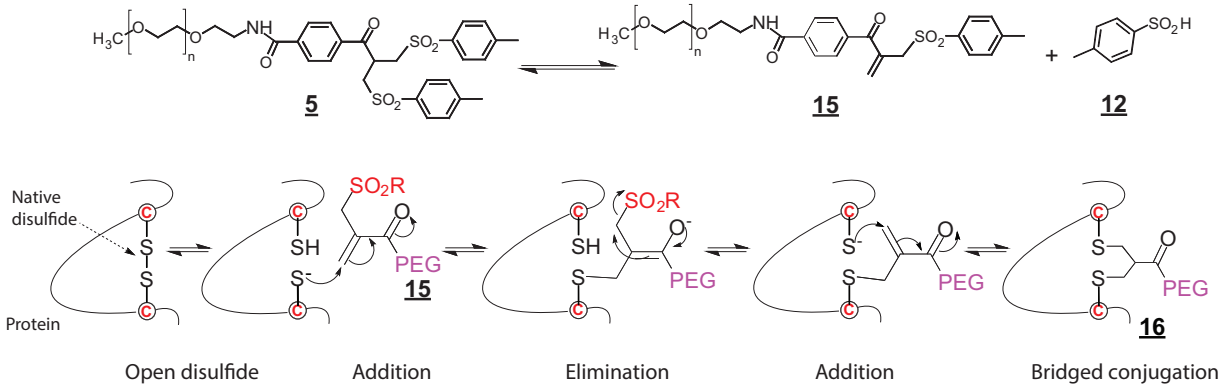

Figure 5. Conjugation by bis-alkylation to re-bridge disulfides using an addition-elimination mechanism. 5 PEG-bis-sulfone reagent, 12 sulfinic acid leaving group, 15 PEG-mono-sulfone enone, 16 final bis-alkylated conjugate

PEGylation Using a PEG-aldehyde Reagent

Reductive amination is considered only to be broadly site-specific for the *N*-terminal amino group of a protein. Optimization of the reaction conditions (e.g., pH, reagent stoichiometry, concentration of protein and reagent) is required for each protein in an effort to exploit the difference in the pK_a of the amine of the *N*-terminal amino acid compared to other amines often present in a protein to achieve high-yielding and homogeneous imine formation in an aqueous medium.

1. A stoichiometric excess 5–10 equivalents of a PEG-aldehyde reagent **Figure 2-2** is usually used. As an example,[28] to *N*-terminally PEGylate a protein, dissolve the protein in solution (1.7 mg/mL, 20 mM sodium acetate, pH 4.5, or a suitable buffer based on stability and solubility of the protein), add the PEG-aldehyde reagent **Figure 2-2** (5–10 equiv.) and $NaCNBH_3$ (40 mM) (**Aldrich Prod. No.** 156159). Then incubate the mixture for 16–24 h at ambient temperature (18–23 °C).
2. The mono-PEGylated protein can be purified by cation-exchange chromatography (e.g., use a linear salt gradient in 20 mM aqueous sodium acetate, pH 4.0). Fractions can be analyzed by SDS-PAGE and the purest fractions then pooled.

References

(1) (a) Ezan, E. *Adv. Drug Del. Rev.* **2013**, *65* (8), 1065–1073; (b) Ezan, E.; Becher, F.; Fenaille, F. *Expert Opin. Drug Metab. Toxicol.* **2014**, *10* (8), 1079–1091; (c) Shi, S. *Current Drug Metabolism* **2014**, *15* (3), 271–290.

(2) Kontermann, R. E. *Curr. Opin. Biotechnol.* **2011**, *22* (6), 868–876.

(3) Elliott, S.; Lorenzini, T.; Asher, S.; Aoki, K.; Brankow, D.; Buck, L.; Busse, L.; Chang, D.; Fuller, J.; Grant, J. *Nat. Biotechnol.* **2003**, *21* (4), 414–421.

(4) Mitragotri, S.; Burke, P. A.; Langer, R. *Nat. Rev. Drug Discovery* **2014**, *13* (9), 655–672.

(5) (a) Bailon, P.; Won, C. *Expert Opin. Drug Deliv.* **2009**, *6* (1), 1–16; (b) Pasut, G.; Veronese, F. M. *J. Controlled Release* **2012**, *161* (2), 461–472.

(6) (a) Abuchowski, A.; van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252* (11), 3578–3581; (b) Abuchowski, A.; McCoy, J.; Palczuk, N. C.; van Es, T.; Davis, F. F. *J Biol. Chem.* **1977**, *252* (11), 3582–3586.

(7) Annibali, V.; Mechelli, R.; Romano, S.; Buscarinu, M. C.; Fornasiero, A.; Umeton, R.; Ricigliano, V. A. G.; Orzi, F.; Coccia, E. M.; Salvetti, M.; Ristori, G. *Cytokine Growth Factor Rev.* **2015**, *26* (2), 221–228.

(8) Wadhwa, M.; Bird, C.; Dougall, T.; Rigsby, P.; Bristow, A.; Thorpe, R. *J. Immunol. Methods* **2015**, *416* (C), 17–28.

(9) Li, W.; Zhan, P.; De Clercq, E.; Lou, H.; Liu, X. *Prog. Polym. Sci.* **2012**, *38* (3-4), 421–444.

(10) (a) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Dis.* **2003**, *2* (3), 214–221; (b) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. *Adv. Drug Del. Rev.* **2003**, *55*, 217–250; (c) Stephanopoulos, N.; Francis, M. B. *Nat. Chem. Biol.* **2011**, *7* (12), 876–884; (d) Nischan, N.; Hackenberger, C. P. R. *J. Org. Chem.* **2014**, *79* (22), 10727–10733; (e) Obermeyer, A. C.; Olsen, B. D. *ACS Macro Lett.* **2015**, *4* (1), 101–110; (f) Boutureira, O.; Bernardes, G. J. L. *Chem. Rev* **2015**, *115* (5), 2174–2195.

(11) (a) Diethelm, S.; Schafroth, M. A.; Carreira, E. M. *Org. Lett.* **2014**, *16* (15), 3908–3911; (b) Rabanel, J.-M.; Hildgen, P.; Banquy, X. *J. Controlled Release* **2014**, *185* (C), 71–87.

(12) (a) Agarwal, P.; Bertozzi, C. R. *Bioconjugate Chem.* **2015**, *26* (2), 176–192; (b) Badescu, G.; Bryant, P.; Bird, M.; Henseleit, K.; Swierkosz, J.; Parekh, V.; Tommasi, R.; Pawlisz, E.; Jurlewicz, K.; Farys, M.; Camper, N.; Sheng, X.; Fisher, M.; Grygorash, R.; Kyle, A.; Abhilash, A.; Frigerio, M.; Edwards, J.; Godwin, A. *Bioconjugate Chem.* **2014**, *25* (6), 1124–1136.

(13) (a) Veronese, F.; Monfardini, C.; Caliceti, P. *J. Controlled Release* **1996**, *40*, 199–209; (b) Zhang, X.; Wang, H.; Ma, Z.; Wu, B. *Expert Opin. Drug Metab. Toxicol.* **2014**, *10* (12), 1691–1702; (c) Baumann, A.; Tuerck, D.; Prabhu, S.; Dickmann, L.; Sims, J. *Drug Discovery Today* **2014**, *19* (10), 1623-1631; (d) Verhoef, J. J. F.; Carpenter, J. F.; Anchordoquy, T. J.; Schellekens, H. *Drug Discovery Today* **2014**, *19* (12), 1945–1952.

(14) Rajan, R. S.; Li, T.; Aras, M.; Sloey, C.; Sutherland, W.; Arai, H.; Briddell, R.; Kinstler, O.; Lueras, A. M. K.; Zhang, Y.; Yeghnazar, H.; Treuheit, M.; Brems, D. N. *Protein Sci.* **2006**, *15* (5), 1063–1075.

(15) (a) Kubetzko, S.; Sarkar, C. A.; Plückthun, A. *Mol. Pharmacol.* **2005**, *68* (5), 1439–1454; (b) Khalili, H.; Godwin, A.; Choi, J.; Lever, R.; Brocchini, S. *Bioconjugate Chem.* **2012**, *23* (11), 2262–2277.

(16) Gonnelli, M.; Strambini, G. B. *Biochim. Biophys. Acta, Proteins Proteomics* **2009**, (1794), 569–576.

(17) Duncan, R.; Gaspar, R. *Mol. Pharmaceutics* **2011**, *8* (6), 2101–2141.

(18) (a) Schellekens, H.; Hennink, W. E.; Brinks, V. *Pharm. Res.* **2013**, *30* (7), 1729–1734; (b) Garay, R. P.; El-Gewely, R.; Armstrong, J. K.; Garratty, G.; Richette, P. *Expert Opin. Drug Delivery* **2012**, *9* (11), 1319–1323.

(19) (a) Rudmann, D. G.; Alston, J. T.; Hanson, J. C.; Heidel, S. *Toxicol. Pathol.* **2013**, *41* (7), 970–983; (b) Bendele, A.; Seely, J.; Richey, C.; Sennello, G.; Shopp, G. *Toxicol. Sci.* **1998**, *42* (2), 152–157.

(20) Schellekens, H. *Clin. Thera.* **2002**, *24* (11), 1720–1740.

(21) Webster, R.; Didier, E.; Harris, P.; Siegel, N.; Stadler, J.; Tilbury, L.; Smith, D. *Drug Metabol. Disposition* **2007**, *35* (1), 9–16.

(22) Gorovits, B.; Wakshull, E.; Pillutla, R.; Xu, Y.; Manning, M. S.; Goyal, J. *J. Immunol. Methods* **2014**, *408* (C), 1–12.

(23) Pasut, G. *Polymers* **2014**, *6* (1), 160–178.

(24) Luxenhofer, R.; Han, Y.; Schulz, A.; Tong, J.; He, Z.; Kabanov, A. V.; Jordan, R. *Macromol. Rapid Commun.* **2012**, *33* (19), 1613–1631.

(25) Lewis, A.; Tang, Y.; Brocchini, S.; Choi, J.-w.; Godwin, A. *Bioconjugate Chem.* **2008**, *19* (11), 2144–2155.

(26) DeAngelis, P. L. *Expert Opin. Drug Delivery* **2015**, *12* (3), 349-352.

(27) Liebner, R.; Mathaes, R.; Meyer, M.; Hey, T.; Winter, G.; Besheer, A. *Eur. J. Pharm. Biopharm.* **2014**, *87* (2), 378–385.

(28) Podobnik, B.; Helk, B.; Smilović, V.; Škrajnar, Š.; Fidler, K.; Jevševar, S.; Godwin, A.; Williams, P. *Bioconjugate Chem.* **2015**, *26* (3), 452–459.

(29) (a) Soundrarajan, N.; Sokalingam, S.; Raghunathan, G.; Budisa, N.; Paik, H.-J.; Yoo, T. H.; Lee, S.-G. *PLoS ONE* **2012**, *7* (10), e46741–9; (b) Cong, Y.; Pawlisz, E.; Bryant, P.; Balan, S.; Laurine, E.; Tommasi, R.; Singh, R.; Dubey, S.; Peciak, K.; Bird, M.; Sivasankar, A.; Swierkosz, J.; Muroni, M.; Heidelberger, S.; Farys, M.; Khayrzad, F.; Edwards, J.; Badescu, G.; Hodgson, I.; Heise, C.; Somavarapu, S.; Liddell, J.; Powell, K.; Zloh, M.; Choi, J.-w.; Godwin, A.; Brocchini, S. *Bioconjugate Chem.* **2012**, *23* (2), 248–263.

(30) (a) Fee, C.; Van Alstine, J. *Methods Biochem. Anal.* **2011**, *54*, 339–362; (b) Payne, R. W.; Murphy, B. M.; Manning, M. C. *Pharm. Dev. Technol.* **2011**, *16* (5), 423–440; (c) Pfister, D.; Morbidelli, M. *J. Controlled Release* **2014**, *180* (C), 134–149.

(31) (a) Alley, S. C.; Benjamin, D. R.; Jeffrey, S. C.; Okeley, N. M.; Meyer, D. L.; Sanderson, R. J.; Senter, P. D. *Bioconjugate Chem.* **2008**, *19*, 759–765; (b) Baldwin, A. D.; Kiick, K. L. *Polym. Chem.* **2012**, *4* (1), 133; (c) Zhang, C.; Liu, Y.; Feng, C.; Wang, Q.; Shi, H.; Zhao, D.; Yu, R.; Su, Z. *Electrophoresis* **2014**, *36* (2), 371–374; (d) Shen, B.-Q.; Xu, K.; Liu, L.; Raab, H.; Bhakta, S.; Kenrick, M.; Parsons-Reponte, K. L.; Tien, J.; Yu, S.-F.; Mai, E.; Li, D.; Tibbitts, J.; Baudys, J.; Saad, O. M.; Scales, S. J.; McDonald, P. J.; Hass, P. E.; Eigenbrot, C.; Nguyen, T.; Solis, W. A.; Fuji, R. N.; Flagella, K. M.; Patel, D.; Spencer, S. D.; Khawli, L. A.; Ebens, A.; Wong, W. L.; Vandlen, R.; Kaur, S.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Junutula, J. R. *Nat. Biotechnol.* **2012**, *30* (2), 184–189.

(32) (a) Lyon, R. P.; Setter, J. R.; Bovee, T. D.; Doronina, S. O.; Hunter, J. H.; Anderson, M. E.; Balasubramanian, C. L.; Duniho, S. M.; Leiske, C. I.; Li, F.; Senter, P. D. *Nat. Biotechnol.* **2014**, 1–7; (b) Fontaine, S. D.; Reid, R.; Robinson, L.; Ashley, G. W.; Santi, D. V. *Bioconjugate Chem.* **2015**, *26* (1), 145–152.

(33) (a) Toda, N.; Asano, S.; Barbas, C. F. *Angew. Chem.* **2013**, *25*, 12592–12596; (b) Patterson, J. T.; Asano, S.; Li, X.; Rader, C.; Barbas III, C. F. *Bioconjugate Chem.* **2014**, *25* (8), 1402–1407.

(34) Badescu, G.; Bryant, P.; Swierkosz, J.; Khayrzad, F.; Pawlisz, E.; Farys, M.; Cong, Y.; Muroni, M.; Rumpf, N.; Brocchini, S.; Godwin, A. *Bioconjugate Chem.* **2014**, *25* (3), 460–469.

(35) Mitchell, T.; Chao, G.; Sitkoff, D.; Lo, F.; Monshizadegan, H.; Meyers, D.; Low, S.; Russo, K.; DiBella, R.; Denhez, F.; Gao, M.; Myers, J.; Duke, G.; Witmer, M.; Miao, B.; Ho, S. P.; Khan, J.; Parker, R. A. *J. Pharmacol. Exp. Ther.* **2014**, *350* (2), 412–424.

(36) Shaunak, S.; Godwin, A.; Choi, J. W.; Balan, S.; Pedone, E.; Vijayarangam, D.; Heidelberger, S.; Teo, I.; Zloh, M.; Brocchini, S. *Nat. Chem. Bio.* **2006**, 312–313.

(37) Balan, S.; Choi , J. W.; Godwin, A.; Teo, I.; Laborde, C. M.; Heidelberger, S.; Zloh, M.; Shaunak, S.; Brocchini, S. Bioconjugate Chem. **2007**, *18* (1), 61–76.

(38) Jones, M. W.; Strickland, R. A.; Schumacher, F. F.; Caddick, S.; Baker, J. R.; Gibson, M. I.; Haddleton, D. M. *J. Am. Chem. Soc.* **2012**, *134* (3), 1847–1852.

(39) Nunes, J. P. M.; Morais, M.; Vassileva, V.; Robinson, E.; Rajkumar, V. S.; Smith, M. E. B.; Pedley, R. B.; Caddick, S.; Baker, J. R.; Chudasama, V. *Chemical Communications* **2015**, *51* (53), 10624–10627.

(40) Duncan, R. *J. Controlled Release* **2014**, *190* (C), 371–380.

(41) Borchmann, D. E.; Carberry, T. P.; Weck, M. *Macromol. Rapid Commun.* **2013**, *35* (1), 27–43.

(42) Lawrence, P. B.; Gavrilov, Y.; Matthews, S. S.; Langlois, M. I.; Shental-Bechor, D.; Greenblatt, H. M.; Pandey, B. K.; Smith, M. S.; Paxman, R.; Torgerson, C. D.; Merrell, J. P.; Ritz, C. C.; Prigozhin, M. B.; Levy, Y.; Price, J. L. *J. Am. Chem. Soc.* **2014**, *136* (50), 17547–17560.

(43) Brocchini, S.; Balan, S.; Godwin, A.; Choi, J. W.; Zloh, M.; Shaunaik, *Nat. Protoc.* **2006**, *1* (5), 2241–2252.

Monofunctional mPEGs

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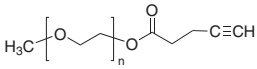
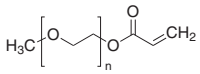
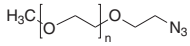
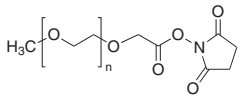
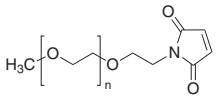
ω-end	Structure	Molecular Weight	Prod. No.
Acetylene		average M_n 2,000	699802-500MG
Acrylate		average M_n 2,000	730270-1G
		average M_n 5,000	730289-1G
Azide		average M_n 1,000	733407-1G
		average M_n 2,000	689807-250MG 689807-1G
		average M_n 5,000	689475-250MG 689475-1G
		average M_n 10,000	726168-250MG
		average M_n 20,000	726176-250MG
NHS ester		average M_n 5,000	85973-1G
Maleimide		average M_n 750	712558-250MG
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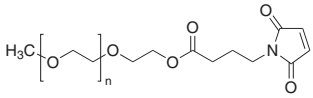
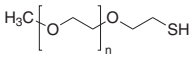
		average M_n 10,000	712469-250MG
		average M_n 2,000	731765-1G 731765-5G
SH		average M_n 800	729108-1G 729108-5G
		average M_n 2,000	729140-1G 729140-5G
		average M_n 6,000	729159-1G 729159-5G

Controlled Release | Targeted Delivery | Solubility Enhancement

POLY(2-OXAZOLINE)S FOR DRUG DELIVERY



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Introduction

Poly(2-oxazoline)s (POx) were discovered in 1966[1,2] but have only recently gained substantial attention as a biomaterial, especially for the development of drug delivery systems (DDS) and polymer therapeutics.[3] One reason for the late recognition of POx in the biomedical field was a lack of commercially available monomers and well-defined, functionalized polymers, while other suitable polymer platforms such as poly(ethylene glycol) (PEG) and Pluronic® block copolymers were readily available from commercial sources. As a consequence, PEG became the most widely used polymer therapeutic and is classified as "Generally Recognized as Safe" (GRAS) by the FDA. However, recent reports on an "accelerated blood clearance" (ABC) effect of PEGylated therapeutics,[4] as well as the rather limited chemical variability of the polymer, has helped make the case for developing suitable alternative polymer platforms. POx has the potential to provide such an alternative in the case of the failure of a PEG-based system. Moreover, POx has the potential to offer more than merely a replacement to PEG because it provides a more versatile chemistry and the potential to tune the "hydrophilic lipophilic balance" (HLB) of amphiphilic POx copolymers.[3,5]

Chemistry

POx is synthesized by living cationic ring-opening polymerization (LCROP) of 2-oxazolines, typically with alkyl tosylates or triflates as initiators and various nucleophiles (amines, carboxylates, etc.) as terminating agents. The LCROP of 2-oxazolines is relatively slow but proceeds reasonably well at T >40 °C to produce well-defined linear polymers of low dispersity (PDI <1.05–1.3), controllable compositions (random, gradient, and block copolymers) and with end groups that can be quantitatively defined by the initiation and termination reaction. Among other factors, the solvent polarity and nature of the counter ion determine the equilibrium and thus, the "livingness" of the LCROP. A general reaction scheme is given in **Figure 1**.

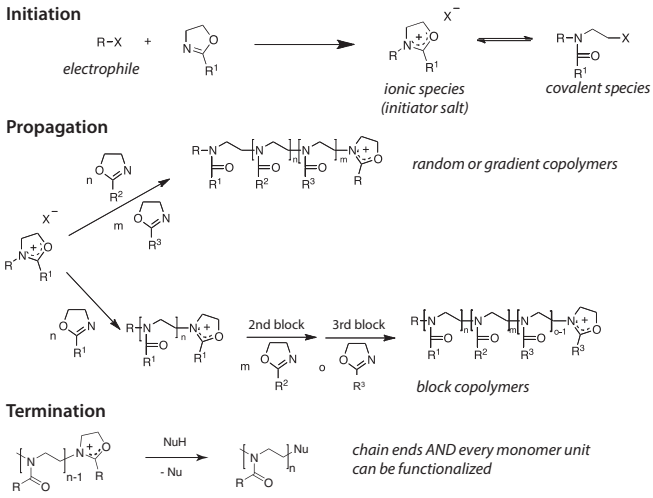


Figure 1. General reaction scheme of the living ring-opening polymerization of 2-oxazolines.

Biocompatibility of POx

The current interest in POx as an alternative polymer platform to PEG is largely based on recent reports on its exceptional behavior in complex biological environments. Zalipsky et al.[6] first discovered the so-called "stealth behavior" of hydrophilic POx (poly(2-methyl- and 2-ethyl-2-oxazoline) (PMeOx, PEtOx)) that leads to long blood circulation times in animals. Later, Luxenhofer et al.[7] found that upon injection in rodents, hydrophilic POx disperses rapidly throughout the entire organism (except the brain) and shows very little unspecific organ deposition and rapid renal excretion. Amphiphilic POx of various compositions display no cytotoxicity, low complement activation *in vitro*[8,9] and animal testing indicates no adverse properties from neutral hydrophilic or amphiphilic POx. Moreover, depending on the amphiphilic contrast of POx, the cell-uptake can be surprisingly fast even at low polymer concentrations.[8]

POx Conjugates

One advantage of POx chemistry is that it allows for the definition of the physical and chemical properties of the final polymer over a very broad range simply by the variation of the monomer side chain (2-substitution). This enables the introduction of various chemical functional groups for later polymer ligation. Among the first functional groups introduced directly onto POx was the alkyne moiety for the subsequent polymer analog CuAAC click chemistry.[10–12] Recently, other "click reactions" such as the "thiol-ene click" have been successfully added to functional POx;[13–16] next-generation biomaterials will include POx conjugates that incorporate small molar mass drugs and proteins.[17–21] Modification of proteins with amphiphilic POx block copolymers imparts these proteins not only with stealth properties and long circulation, but also with the ability to cross biological barriers; for example, increase protein uptake in cells or transport to the brain.[18] For a more exhaustive list of reported end- and side-functionalized POx, there are several recent reviews.[3,22–25]

POx Polyplexes

POx has long been used for the preparation of polyplexes for gene delivery because linear poly(ethylene imine) (PEI) is derived from linear POx (poly(2-ethyl-2-oxazoline)) by hydrolysis. Recently, cationic POx prepared from protected amine-functionalized 2-oxazolines[26] or via thiol-ene click reactions[27] was investigated as a nonviral vector for use in gene therapy.[27,28] Schubert et al.[27] generated a library of various cationic POx and identified one variant that had similar transfection efficiency to linear poly(ethyleneimine) in HEK cells. Kabanov et al.[28] reported transfection of very-hard-to-transfect cells using POx-based polyplexes in combination with Pluronic® block copolymers as the transfection enhancer. Interestingly, in the latter report an intriguing amount of low plasma protein binding with polyplexes was observed, which highlights the stealth properties of hydrophilic POx and is of particular interest for targeted gene delivery. Additionally, various other polycations were combined with hydrophilic POx to create nonviral vectors, taking advantage of the stealth properties of the hydrophilic POx shell around the polyplex core.[3]

Amphiphilic POx as Micellar DDS

As mentioned previously, the physical properties of POx can be fine-tuned through the 2-substitution of the monomer. This can be used to vary the solubility of POx in water over a wide range and, in combination with (block) copolymerization, results in highly defined amphiphilic POx. In contrast to Pluronic® block copolymers, the amphiphilic motif is not only found along the polymer backbone but a second amphiphilic motif is located within each monomer unit. Thus, hydrophobic POx is actually a polysoap with a hydrophobic pendant group linked via a polar, hydrophilic amide group to the polymer chain (**Figure 2**).

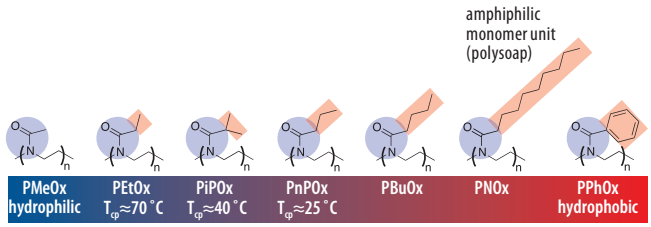


Figure 2. Series of hydrophilic to hydrophobic poly(2-oxazoline)s from the pure hydrophilic PMeOx to PEtOx (slightly amphiphilic and similar to PEG) over thermo-sensitive POx (having a critical solution temperature as indicated), to the first water-insoluble PBuOx. Because of the amide group linking the side to the main chain, even water-insoluble POx are amphiphilic (non-ionic polysoap).

Short (<C4) pendant chains result in water-soluble polymers while longer aliphatic or aromatic side chains result in hydrophobic polymers (**Figure 2**). Similar to other water-soluble polymers, aqueous POx solutions exhibit reversible phase-transitions (C2 and C3 side chains) which renders POx as an ideal candidate for the design of "smart polymer materials."[29–32] POx displays a very narrow phase transition behavior of only 1–2 K and low hysteresis.

In principle, all combinations of hydrophilic and hydrophobic monomer units are possible in random, gradient, and multiblock copolymers. This results in a series of polymeric amphiphiles similar to Pluronic® block copolymers. In water the POx amphiphiles assemble into polymeric micelles of distinct shape and typically small size, with a very low critical micellar concentration (CMC ≈ 10^{-5} M). Thus, amphiphilic POx is an ideal system for the solubilization, formulation, and delivery of strongly hydrophobic drugs.

The formulation of paclitaxel (PTX) in a P(MeOx-BuOx-MeOx) triblock copolymer is one outstanding example of amphiphilic POx in micellar drug delivery. PTX is one of the most frequently used anticancer drugs that suppresses cell division by association to microtubules and leads to apoptosis. Like many other small-molecule drugs, PTX suffers from very low water solubility, only 1 µg/mL. In currently used commercial PTX formulations, the ratio of excipient to PTX is around 100:1 (Taxol®) or 10:1 (Abraxane®). However, the triblock POx formulation P(MeOx-BuOx-MeOx) was found to solubilize PTX at a ratio of POx:PTX of 1:1 (50 wt% drug loading instead of 1 or 10 wt%, respectively). Moreover, drug concentrations as high as 50 mg/mL were stable in the injectable triblock formulation, increasing drug solubility by a factor of 50,000.[9,33,34] This unparalleled high drug loading capacity initiated a series of studies to solubilize various other drugs in a variety of amphiphilic POx.[33,35–38] PBuOx containing block copolymers were the most effective for drug solubilization, while the use of a more hydrophobic PNOx block resulted in acceptable but not extraordinary drug loadings. The high drug loading of PBuOx copolymers can be associated with the hydrophobic and polar interactions between the polymer excipient and the drug. Flexible composition of the polymer carrier and optimization of its interaction with the drug are both critical to efficient drug solubilization and formulation. While maintaining high drug loading capacity is important, it is also critical to maintain simplicity of the formulation procedure.

Typically, the drug and the polymer are solubilized in a common solvent, and the solvent is evaporated until a thin film remains and then water or buffer is added. For most drug-POx combinations, this "thin film method" results in clear and stable micellar solutions with different maximum drug loadings depending on the formulation. The formulations can typically be reconstructed multiple times without noticeable differences in the micellar characteristics. The simple procedure is outlined in **Figure 3**.

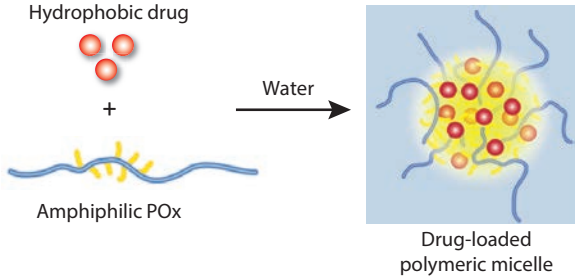


Figure 3. Preparation of drug loaded polymeric micelles with an amphiphilic triblock POx as the only excipient. (For the general procedure, see A. Du, M. Stenzel in this publication.)

Conclusions

Poly(2-oxazoline)s (POx) have recently emerged as a popular candidate for the development of new polymer therapeutics due largely to its biocompatibility and versatility in forming well-defined POx-drug and POx-protein conjugates, polyplexes, and polymeric micelles from amphiphilic POx. With preclinical data recently completed, the translation of POx-based polymer therapeutics into clinical trials is soon expected.

Method: Block Copolymerization of 2-Oxazolines

The polymerization is carried out under dry inert conditions with freshly distilled and degassed solvents and monomers (Schlenk conditions).

Synthesis of P(MeOx$_{27}$-BuOx$_{12}$-MeOx$_{27}$)

1. At room temperature, 32.2 mg (0.2 mmol, 1 eq) of methyltriflate (MeOTf) (**Aldrich Prod. No.** 164283) and 440 mg (5.17 mmol, 26 eq) of 2-methyl-2-oxazoline (MeOx) (**Aldrich Prod. No.** 137448) are dissolved in 3 mL acetonitrile.
2. The mixture is heated to 130 °C (microwave, 150 W) for 15 min. After equilibration to room temperature, the monomer for the second block, 2-butyl-2-oxazoline (256 mg, 2.01 mmol, 10 eq) (**Aldrich Prod. No.** 799637) is added under dry nitrogen and heated again for 15 min.
3. For the third block, the procedure is repeated with 442 mg MeOx (5.19 mmol, 26 eq). The polymerization is terminated at room temperature by the addition of 0.1 mL piperidine (1.01 mmol, 5 eq) (**Sigma-Aldrich Prod. No.** 104094) and stirring overnight.
4. Finally, an access of K_2CO_3 is added and the mixture is allowed to stir for several hours.
5. After filtration, the solvent is removed and the polymer precipitated (chloroform to diethylether, 1:10) collected and freeze-dried. Expected yield: ≥90%, PDI ≤1.2.

Preparation of Drug-loaded POx Micelles by Thin Film Method/Co-solvent Evaporation

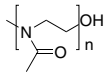
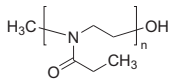
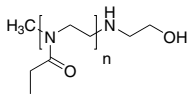
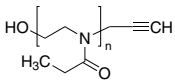
1. POx and drug are dissolved in a minimum amount of a common solvent (e.g., ethanol).
2. The solvent is slowly evaporated under reduced pressure and/or mild heat until a thin, clear film is obtained.
3. Water, buffer, or other aqueous solvent is added dropwise (possibly mild heat, 50–60 °C, can be applied) until a clear solution is obtained.
4. Drug loading capacity is determined by HPLC.

References

(1) Tomalia, D. A.; Sheetz, D. P. *Journal of Polymer Science Part A-1: Polymer Chemistry* **1966**, *4*, 2253–2265.
(2) Aoi, K.; Okada, M. *Prog. Polym. Sci.* **1996**, *21*, 151–208.
(3) Luxenhofer, R.; Han, Y.; Schulz, A.; Tong, J.; He, Z.; Kabanov, A. V.; Jordan, R. *Macromol. Rapid Commun.* **2012**, *33*, 1613–1631.
(4) Yang, Q.; Lai, S. K. *WIRE-Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2015**, *7* (5) 655–77
(5) Sedlacek, O.; Monnery, B. D.; Filippov, S. K.; Hoogenboom, R.; Hruby, M. *Macromol. Rapid Commun.* **2012**, *33*, 1648–1662.
(6) Woodle, M. C.; Engbers, C. M.; Zalipsky, S. *Bioconjugate Chem.* **1994**, *5*, 493–496.
(7) Gaertner, F. C.; Luxenhofer, R.; Blechert, B.; Jordan, R.; Essler, M. *J. Controlled Release* **2007**, *119*, 291–300.
(8) Luxenhofer, R.; Sahay, G.; Schulz, A.; Alakhova, D.; Bronich, T. K.; Jordan, R.; Kabanov, A. V. *J. Controlled Release* **2011**, *153*, 73–82.
(9) Luxenhofer, R.; Schulz, A.; Roques, C.; Li, S.; Bronich, T. K.; Batrakova, E. V.; Jordan, R.; Kabanov, A. V. *Biomaterials* **2010**, *31*, 4972–4979.
(10) Luxenhofer, R.; Jordan, R. *Macromolecules* **2006**, *39*, 3509–3516.
(11) Manzenrieder, F.; Luxenhofer, R.; Retzlaff, M.; Jordan, R.; Finn, M. G. *Angew. Chem. Int. Ed.* **2011**, *50*, 2601–2605.
(12) Fijten, M. W. M.; Haensch, C.; van Lankvelt, B. M.; Hoogenboom, R.; Schubert, U. S. *Macromol. Chem. Phys.* **2008**, *209*, 1887–1895.
(13) Cortez, M. A.; Grayson, S. M. *Macromolecules* **2010**, *43*, 4081–4090.
(14) Kempe, K.; Hoogenboom, R.; Jaeger, M.; Schubert, U. S. *Macromolecules* **2011**, *44*, 6424–6432.
(15) Dargaville, T. R.; Forster, R.; Farrugia, B. L.; Kempe, K.; Voorhaar, L.; Schubert, U. S.; Hoogenboom, R. *Macromol. Rapid Commun.* **2012**, *33*, 1695–1700.
(16) Schubert, U. S.; Hartlieb, M.; Kempe, K. *Journal of Materials Chemistry B* **2014**, *3*, 526–538.
(17) Tong, J.; Yi, X.; Luxenhofer, R.; Banks, W. A.; Jordan, R.; Zimmermann, M. C.; Kabanov, A. V. *Mol. Pharmaceutics* **2013**, *10*, 360–377.
(18) Tong, J.; Luxenhofer, R.; Yi, X.; Jordan, R.; Kabanov, A. V. *Mol. Pharmaceutics* **2010**, *7*, 984–992.
(19) Mero, A.; Fang, Z.; Pasut, G.; Veronese, F. M.; Viegas, T. X. *J. Controlled Release* **2012**, 159, 353–361.
(20) Harris, J. M.; Bentley, M. D.; Viegas, T. X. *8th Intern. Symp. on Polymer Therapeutics: From Laboratory to Clinical Practice* **2010**, 19.
(21) Viegas, T. X.; Bentley, M. D.; Harris, J. M.; Fang, Z.; Yoon, K.; Dizman, B.; Weimer, R.; Mero, A.; Pasut, G.; Veronese, F. M. *Bioconjug. Chem.* **2011**, *22*, 976–986.
(22) Lava, K.; Verbraeken, B.; Hoogenboom, R. *Eur. Polym. J.* **2015**, *65*, 98–111.
(23) Rossegger, E.; Schenk, V.; Wiesbrock, F. *Polymers* **2013**, *5*, 956–1011.
(24) Luxenhofer, R.; Jordan, R. *Materials Matter* **2013**, *8*, 70–73.
(25) de la Rosa, V. R. *J. Mater. Sci. Mater. Med.* **2014**, *25*, 1211–1225.
(26) Cesana, S.; Auernheimer, J.; Jordan, R.; Kessler, H.; Nuyken, O. *Macromol. Chem. Phys.* **2006**, *207*, 183–192.
(27) Rinkenauer, A. C.; Tauhardt, L.; Wendler, F.; Kempe, K.; Gottschaldt, M.; Traeger, A.; Schubert, U. S. *Macromol. Biosci.* **2015**, *15* (3), 414–425
(28) He, Z.; Miao, L.; Jordan, R.; S-Manickam, D.; Luxenhofer, R.; Kabanov, A. V. *Macromol. Biosci.* **2015**, *15*, in print.
(29) Huber, S.; Jordan, R. *Colloid Polym. Sci.* **2008**, *286*, 395–402.
(30) Park, J. S.; Kataoka, K. *Macromolecules* **2007**, *40*, 3599–3609.
(31) Hoogenboom, R.; Thijs, H. M. L.; Jochems, M. J. H. C.; van Lankvelt, B. M.; Fijten, M. W. M.; Schubert, U. S. *Chem. Commun.* **2008**, *41*, 5758–5759
(32) Weber, C.; Hoogenboom, R.; Schubert, U. S. *Prog. Polym. Sci.* **2012**, *37*, 686–714.
(33) Schulz, A.; Jaksch, S.; Schubel, R.; Wegener, E.; Di, Z.; Han, Y.; Meister, A.; Kressler, J.; Kabanov, A. V.; Luxenhofer, R.; Papadakis, C. M.; Jordan, R. *ACS Nano* **2014**, *8*, 2686–2696.
(34) Kabanov, A.; Jordan, R.; Luxenhofer, R., PCT Appl. EP2009/004655, US 61/133,154, US 61/134,209, RU 2523714, JP 5671457.
(35) Tong, J.; Zimmermann, M. C.; Li, S.; Yi, X.; Luxenhofer, R.; Jordan, R.; Kabanov, A. V. *Biomaterials* **2011**, *32*, 3654–3665.
(36) Han, Y.; He, Z.; Schulz, A.; Bronich, T. K.; Jordan, R.; Luxenhofer, R.; Kabanov, A. V. *Mol. Pharmaceutics* **2012**, *9*, 2302–2313.
(37) He, Z.; Schulz, A.; Wan, X.; Seitz, J.; Bludau, H.; Darr, D. B.; Perou, C. M.; Jordan, R.; Ojima, I.; Kabanov, A. V.; Luxenhofer, R. *J. Controlled Release* **2015**, *208*, 67–75.
(38) Seo, Y.; Schulz, A.; Han, Y.; He, Z.; Bludau, H.; Wan, X.; Tong, J.; Bronich, T. K.; Luxenhofer, R.; Jordan, R.; Kabanov, A. V. *Polym. Adv. Technol.* **2015**, *26*, 837–850.

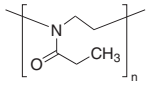
Low PDI Poly(oxazoline)s

For more information on these products, visit aldrich.com/pox.

Name	Structure	Molecular Weight	PDI	Prod. No.
Poly(2-methyl-2-oxazoline), hydroxy terminated		average M_n 2,000	< 1.2 PDI	795275-5G
Poly(2-ethyl-2-oxazoline)		average M_n 5,000	≤ 1.2 PDI	740713-5G
		average M_n 25,000	≤ 1.4 PDI	741884-5G
		average M_n 10,000	< 1.3 PDI	741906-5G
Poly(2-ethyl-2-oxazoline) α-methyl, ω-2-hydroxyethylamine terminated		average M_n 2,000	≤ 1.2 PDI	773360-1G
		average M_n 5,000	< 1.3 PDI	773379-1G
		average M_n 10,000	< 1.3 PDI	773387-1G
Poly(2-ethyl-2-oxazoline), alkyne terminated		average M_n 2,000	≤ 1.1 PDI	747262-1G
		average M_n 5,000	≤ 1.2 PDI	778338-1G

Poly(2-ethyl-2-oxazoline)

For more information on these products, visit aldrich.com/pox.

Name	Structure	Molecular Weight	Prod. No.
Poly(2-ethyl-2-oxazoline)		average M_w 50,000	372846-100G 372846-500G
		average M_w 200,000	372854-100G
		average M_w 500,000	373974-100G 373974-500G

Controlled Release | Targeted Delivery | Solubility Enhancement

POLYOXAZOLINES: AN ALTERNATIVE TO POLYETHYLENE GLYCOL

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Water-soluble, biocompatible polymers are important building blocks in drug delivery formulations, from micelles to drug-polymer conjugates. Polyethylene glycol (PEG) is often considered the gold standard for biocompatible and inert polymers used in drug delivery formulations. However, the growing demand for suitable polymer candidates with a range of material properties to fit the increasing diversity of drugs has generated a need for new biocompatible multifunctional polymers.

Poly(2-ethyl-2-oxazoline)s and poly(2-methyl-2-oxazoline)s are as extensively studied as poly(ethylene glycol) substitutes in a variety of biomedical applications since they share many of the desirable properties of PEG[1–4] but avoid some of their limitations (**Table 1**). PEG has been widely used in biomedical applications because it imparts "stealth" properties to the drug delivery system. PEGylation, the conjugation of PEG to a biopharmaceutical, has helped generate both clinical and market success for a number of drugs. Although PEG has traditionally been considered bio-inert, recently, serum antibodies against PEG have been detected in patients treated with PEG-uricase[5] and PEG-asparaginase.[6] Moreover, in a pre-treatment screening, over 25% of patients had pre-existing anti-PEG antibodies even though they never received prior treatment with PEGylated drugs.[7,8] This may be due to the abundance of PEG used in everything from beauty products to food. Furthermore, PEGylated conjugates can cause kidney cell vacuoluation in animals following repeated administration of PEGylated therapeutics.[9,10]

Poly(2-oxazolines) may be a suitable alternative to PEG due to their biocompatible properties. For example, radiolabeling of polyoxazoline has shown that the polymer is rapidly excreted by the kidneys and shows no accumulation in the body.[11,12] In addition, *in vitro* studies with poly(2-ethyl-2-oxazoline) and poly(2-ethyl-2-oxazoline) block copolymers were reported to be biocompatibile even at higher concentrations than PEG.[13,14] For these reasons, poly(2-oxazolines) are being investigated in a variety of pharmaceutical and medical applications, e.g., drug-polymer conjugates (POZylation),[15,16] micelles,[17] or grafting onto liposomal bilayers.[18]

Table 1. Property comparison between PEG and Poly(2-oxazoline)s*

Poly(2-oxazoline)	PEG
Polymerization	
Easily synthesized with commercially available materials	Challenging polymerization
No peroxide formation	Forms peroxides
No diol impurities	May have up to 6% diol content as an impurity
Polymer Properties	
Low viscosity	High viscosity at high concentrations
Stable at room temperature	Stable at <–20 °C
Non-hygroscopic	–
Drug Delivery Applications	
Not approved by the FDA yet	FDA approved
High drug loading	Low drug loading
Pendant functional groups allow for active targeting by conjugation techniques	Active targeting is reserved for conjugation to end-functional group only
Readily cleared from the body	May accumulate *in vivo*
–	May be immunogenic for a subset of patients
–	Vacuole formation observed

*adapted from Viegas et al.[15]

References

(1) Chapman, R. G.; Ostuni, E.; Takayama, S.; Holmlin, R. E.; Yan, L.; Whitesides, G. M. *J. Am. Chem. Soc.* **2000**, *122*, 8303.
(2) Konradi, R.; Pidhatika, B.; Muhlebach, A.; Textort, M. *Langmuir* **2008**, *24*, 613.
(3) Zalipsky, S., Hansen, C. B.; Oaks, J. M.; Allen, T. M. *J. Pharm. Sci.* **1996**, *85*, 133.
(4) Pidhatika, B.; Rodenstein, M.; Chen, Y.; Rakhmatullina, E.; Muhlebach, A.; Acikgoz, C.; Textor, M.; Konradi, R. *Biointerphases* **2012**, *7*, 1.
(5) Sherman, M. R.; Saifer, M. G.; Perez-Ruiz, F. *Adv. Drug Delivery Rev.* **2008**, *6*, 59–68.
(6) Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J.; Garratty, G. *Cancer* **2007**, *110*, 103–11.
(7) Leger, R. M.; Arndt, P.; Garratty, G.; Armstrong, J. K.; Meiselman, H. J.; Fisher, T. C. *Transfusion* **2001**, *41*, 29S.
(8) Armstrong, J. K.; Leger, R.; Wenby, R. B.; Meiselman, H. J.; Garratty, G.; Fisher, T. C. *Blood* **2003**, *102*, 556A.
(9) Bendele, A.; Seely, J.; Richey, C.; Sennello, G.; Shopp, G. *Toxicol. Sci.* **1998**, *42*, 152–157.
(10) Conover, C.; Lejeune, L.; Linberg, R.; Shum, K.; Shorr, R. G. L. *Immob. Biotechnol.* **1996**, *24*, 599–611.
(11) Goddard, P.; Hutchinson, L. E.; Brown, J.; Brookman, L. J. *J. Controlled Release* **1989**, *10*, 5–16.
(12) Gaertner, F. C.; Luxenhofer, R.; Blechert, B.; Jordan, R.; Essler, M. *J. Controlled Release* **2007**, *119*, 291–300.
(13) Kronek, J.; Kronekova, Z.; Luston, J.; Paulovicova, E.; Paulovicova, L.; Mendrek, B. *J. Mater Sci Mater Med.* **2011**, *22*, 1725–34.
(14) Luxenhofer, R.; Sahay, G.; Schulz, A.; Alakhova, D.; Bronich, T. K.; Jordan, R.; Kabanov, A. V. *J. Controlled Release* **2011**, *53*, 73–82.
(15) Viegas, T.; Bentley, M.; Harris, J.; Fang, Z.; Yoon, K.; Dizman, B.; Weimer, R.; Mero, A.; Paut, G.; Veronese, F. *Bioconjugate Chem.* **2011**, *22*, 976–986.
(16) Mero, A.; Pasut, G.; Dalla, Via L.; Fijten, M. W.; Schubert, U. S.; Hoogenboom, R.; Veronese, F. M. *J. Controlled Release* **2008**, *2*, 87–95.
(17) Wang, C. H.; Hsiue, G. H. *J. Controlled Release* **2005**, *108*, 140–149.
(18) Zalipsky, S.; Hansen, C. B.; Oaks, J. M.; Allen, T. M. *J. Pharm. Sci.* **1996**, *85*, 133–137.

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DENDRITIC POLYESTER SCAFFOLDS:
FUNCTIONAL AND BIOCOMPATIBLE PRECISION POLYMERS FOR DRUG DELIVERY APPLICATIONS


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Introduction

Dendrimers and dendrons are highly branched polymer structures with a precise distribution of functional groups. In contrast to their linear analogs, they are monodisperse in nature and are extremely consistent from batch-to-batch. A broad range of sophisticated applications have capitalized on these dendritic materials including catalysis, optics, and nanomedicine.[1] In nanomedicine, great efforts have been devoted toward the design of selective drug delivery systems and efficient imaging probes.[2] Thus, dendrimers present significant advantages over linear polymers, including the following:

- They are precision nanomedicines carrying an exact number of drugs, all arising from their controlled synthesis and monodisperse nature.
- Their properties can be directly correlated to their structure and present predictable and repeatable pharmacokinetics.
- They can be tuned to present optimal properties such as biodegradability, low toxicity, and water solubility.
- They provide an increased loading capacity, achieved by encapsulation or direct conjugation of drugs with different hydrophilic/hydrophobic properties.
- The drug release profile can selectively be tuned and controlled.
- They are intrinsically highly functional and can be designed to present multipurpose actions (e.g., therapeutic, diagnosis, targeting).

Non-toxic Biodegradable bis-MPA Dendritic Scaffolds

A promising family of dendritic macromolecules is based on the 2,2-bis(methylol)propionic acid (bis-MPA) building block.[3–4] These scaffolds exhibit highly valuable properties for biomedical applications including low or no *in vitro* toxicity, no specific *in vivo* organ accumulation, no immunogenic profile, and biodegradability under physiological conditions.[5–6] Moreover, the use of both the bis-MPA monomer and dendritic macromolecules has increased due to the commercial availability of a wide range of hyperbranched polymers, dendrimers, and dendrons (**Figure 1**). Herein, the most advanced applications of bis-MPA based dendritic materials as drug delivery agents will be summarized.

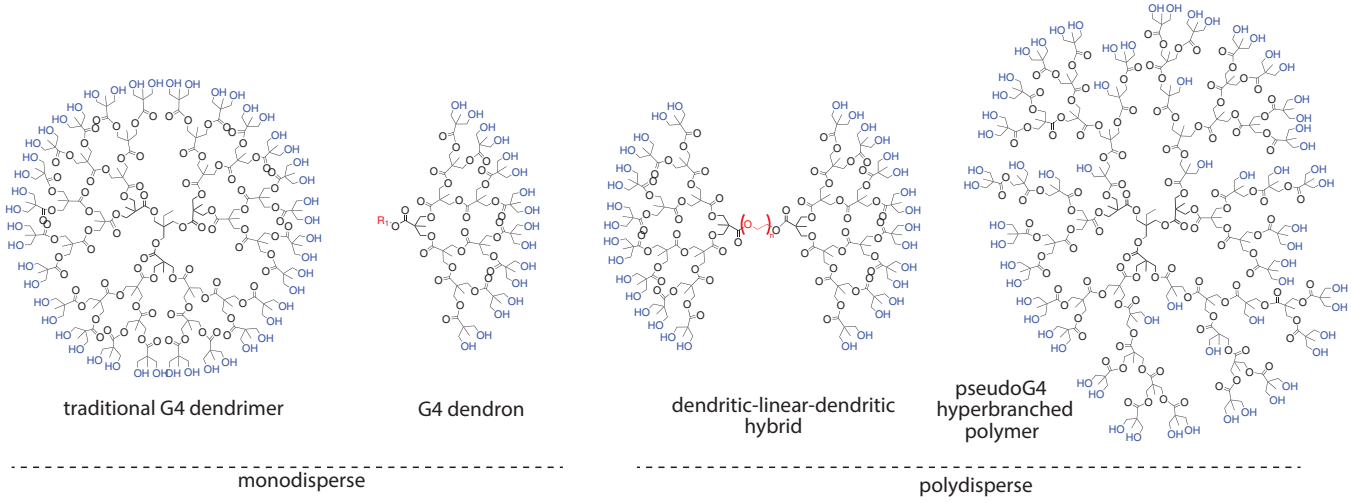

Figure 1. Examples of bis-MPA based dendritic structures of generation 4.

Novel bis-MPA Dendritic Scaffolds for Targeted Drug Delivery

The dendritic family of bis-MPA scaffolds has proven to be an efficient tool for delivery of both physically and covalently attached drugs.

Passive Encapsulators

Bis-MPA dendrimers have been reported as highly effective formulation systems for drugs physically encapsulated in the interior, based on hydrophobic, ionic, or charged-based interactions. They have demonstrated enhancement of the solubility of different chemotherapeutics (e.g., such as doxorubicin,[7–8] camptothecin,[9–10] and paclitaxel[11]) and stabilize colloidal silver particles with antimicrobial properties.[12] The dendritic components act as controlled drug delivery carriers to encapsulate a large amount of the hydrophobic drugs that are released from the nanocarrier to its surroundings upon external stimuli such as changes in pH, temperature, or enzymes. The hybridization of bis-MPA dendritic units with linear polymers such as polyethylene glycol (PEG) imparts stealth properties, increases circulation time, and can endow novel properties such as self-assembly into micelles to improve drug encapsulation.[13–15] One of the most remarkable examples is the theranostic nanoparticles described by Nyström et al.[16] in which star-shaped dendritic-linear hybrids self-assembled into carriers capable of efficiently delivering doxorubicin and acting as *in vivo* contrast agents for ^{19}F-MRI. Analogous hyperbranched polymers also have been widely used as passive encapsulators due to their straightforward preparation and similar behavior to their monodisperse counterparts.[14]

Unimolecular Carriers

Today many pharmaceutically active drugs are intrinsically hydrophobic. Researchers are continually proposing new platforms of biocompatible and unimolecular drug delivery systems that can carry a large cargo of covalently conjugated drugs, along with providing appropriate aqueous solubility and allowing controlled release upon reaching the targeted cells and tissues. Among the many proposed drug delivery systems, monodisperse and highly functional dendritic frameworks based on bis-MPA are the most intriguing. This is due to their structural flawlessness coupled with biocompatibility, biodegradability, and lack of toxicity. Furthermore, careful selection of the conjugation chemistry between the bis-MPA dendritic carrier and the desired cargo enables controlled release of pharmaceutically active drugs. Such controlled release is typically triggered by external stimuli such as variations in pH, intracellular glutathione levels, or the reductive potential in the cell. One of the most advanced studies is based on a bow-tie bis-MPA dendrimer containing degradable linkages of doxorubicin on one side and PEG chains on the other side. This scaffold produced a 9-fold higher specific tumor uptake compared to the free drug in a colorectal xenograft tumor animal model.[17] The attachment of certain active components, such as sugar or peptide moieties,[18–20] leads to unimolecular therapeutic agents that take advantage of the multivalency of the scaffold. One clear example, described by Hawker et al., is the bifunctional bis-MPA dendrimers containing both bioactive mannose and fluorescent coumarin units.[21] The dendritic macromolecules proved to be highly efficient recognition/detection agents for the inhibition of hemagglutination with a 240-fold greater potency than monomeric mannose.

Facile Formulation Protocols

With the wide range of polyester dendrons and dendrimers based on bis-MPA currently available on the market, straightforward reactions like one-step functionalization are aiding in the development of many final sophisticated bioactive materials. The recent development of Fluoride-Promoted Esterification (FPE) through imidazolide-activated compounds acts as a powerful tool for the modification of highly functional polymers under benign and green chemistry conditions.[22] Through this protocol, the hydroxyl[4] or carboxyl[23] periphery of the dendritic scaffolds can be easily functionalized with a large amount of moieties (drugs, targeting moieties, dyes, etc.). A more advanced process called Click chemistry was introduced in 2001 by Prof. K. B. Sharpless.[24] Click chemistry is based on a set of highly reliable and synthetically simple chemical reactions known to proceed in a wide range of solvents, including organic and aqueous conditions with high conversions in short reaction times. In the case of bis-MPA dendritic scaffolds, Click reactions have been used for the successful conjugation of bioactive moieties such as PEG,[14] saccharides,[18–19] peptides, and antibiotics.[19]

The combination of these one-step functionalization protocols allows the straightforward development of advanced dendritic materials. The presence of common "clickable" groups in the core or periphery of the dendritic molecule such as azides (-N_3), acetylenes (-C≡CH), thiols (-SH), and alkenes (-C=CH_2) allows the simple substitution at this position via Click reactions. The multiple hydroxyl or carboxyl groups in the surface are susceptible to modification with a broad range of compounds via the FPE protocol. Typical reaction conditions for the modification of the core and the periphery of the dendritic scaffolds are depicted in **Table 1**.

Table 1. Typical reaction conditions for the peripheral and core functionalization of commercially available bis-MPA based dendritic structures.

		Reaction	——— Conditions ———	
Periphery	-OH -COOH	FPE[22–23]	RCOOH, CDI CsF EtOAc, 50 °C	1.5 eq./OH 0.2 eq./OH
	-≡	CuAAC[25]	R-N_3 $CuSO_4$·$5H_2O$ NaAsc THF:H_2O, r.t.	1.1 eq./ck 0.1 eq./ck 0.3 eq./ck
Core	-OH -COOH	FPE[22]	RCOOH, CDI CsF EtOAc, 50 °C	1.5 eq./OH 0.2 eq./OH
	-N_3 -≡	CuAAC[25]	R-N_3 or R-≡ $CuSO_4$·$5H_2O$ NaAsc THF:H_2O, r.t.	1.1 eq./ck 0.1 eq./ck 0.3 eq./ck
	-= -SH	TEC[25]	R-SH or R-= DMPA THF, r.t.	2.0 eq./ene 0.2 eq./ene hv (365 nm)

Considering these one-step functionalizations, the preparation of advanced homofunctional and heterofunctional bis-MPA dendrimers is currently an easily achievable task. As detailed in **Figure 2**, in a two-step process the most advanced heterofunctional dendrimers can be realized. Nevertheless, the suggested reactions are only guidelines that may need to be adapted based on the functionalization of interest.

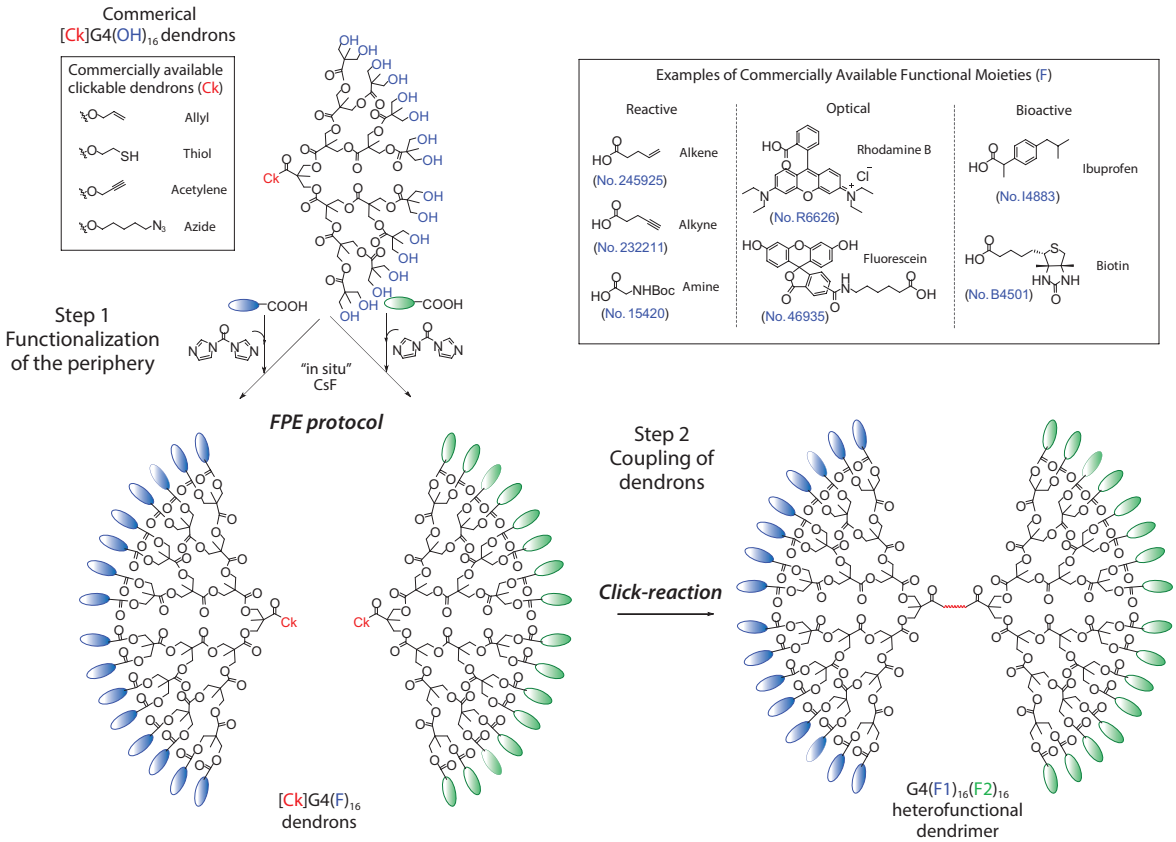

Figure 2. Example of a two-step approach toward bis-MPA based heterofunctional dendrimers.

Summary

Dendritic polymers, including monodisperse dendrimers and dendrons, are by definition the ultimate precision polymers. They are defined as highly branched scaffolds with a large functional group density that can carry a large payload of active drugs. Due to their structurally flawless nature, dendrimers and dendrons belong to a group of the most advanced drug delivery carriers in which batch-to-batch consistency plays a crucial role in future therapeutic breakthroughs. Among the many available dendritic platforms, bis-MPA based polyester scaffolds have attracted the most research interest for biomedical applications. This is mainly due to their structural diversity and simple functionalization protocols along with being biocompatible, biodegradable, and non-toxic. Their commercial availability coupled with the rise of facile post-functionalization protocols such as Click chemistry and FPE reactions gives non-dendrimer specialists the necessary toolbox to custom-build next-generation sophisticated drug delivery systems.

Methods: Dendritic Carriers

The following procedures describe two general protocols for the preparation of dendritic-based drug delivery systems. The suggested reactions are only guidelines that may need to be adapted based on the molecules of interest.

Drug Passive Encapsulators

Drug loading varies significantly depending upon both the drug and the dendrimer structure, making a universal protocol difficult to establish. However, the following is an example that can be used to establish an initial protocol.[8]

1. The dendritic component is dissolved in phosphate buffer saline (PBS).
2. Subsequently, a chloroform solution of the drug is added dropwise under stirring.
3. After evaporation of the chloroform, the free drug is removed by spin filtration.

Drug Unimolecular Carrier

As detailed in **Figure 2**, an advanced heterofunctional dendritic carrier can be easily obtained in a two-step process.

1. **Surface Modification via FPE Protocol**. The molecule of interest (drug, dye, etc.) containing a carboxylic group F-COOH is activated with 1,1´-carbonyldiimidazole (CDI, **Aldrich Prod. No.** 115533) in EtOAc at 50 ºC for 1 h. The *in situ* reaction with the hydroxyl-functional dendron at 50 ºC and CsF (**Aldrich Prod. No.** 20989), and subsequent quenching and washing with $NaHCO_3$ 10%, provides the bifunctional dendron $[Ck]Gn(F)_m$.
2. **Coupling of Dendrons via Click Reactions**. The covalent attachment of two dendrons containing complementary clickable moieties can be achieved through different Click reactions. The most common protocols are as follows:
   - *Click Reaction Between Acetylene and Azide (CuAAC).* To a THF solution of the dendrons, freshly prepared solutions of $CuSO_4 \cdot 5H_2O$ (**Aldrich Prod. No.** 209198) and sodium ascorbate (**Sigma Prod. No.** A7631) are added. The reaction proceeds at room temperature until the total disappearance of the clickable groups' signals. The same reactions also can be accomplished in organic solvents using combinations of CuBr (**Aldrich Prod. No.** 212865) and stabilizing ligands such as tris-(benzyltriazolylmethyl)amine (**Aldrich Prod. No.** 678937), or organic copper complexes such as bromotris(triphenylphosphine) copper(I) (**Aldrich Prod. No.** 572144).
   - *Click Reaction Between Thiol and Alkene (UV-initiated Thiol-ene).* To a deoxygenated MeOH solution of the dendrons, a photoinitiator such as 2,2-dimethoxy-2-phenylacetophenone (**Aldrich Prod. No.** 196118) is added and the mixture is irradiated with UV light at 365 nm until the completion of the reaction.

Both protocols lead to a bifunctional dendrimer $Gn[F1]_m[F2]_n$.

References

(1) Tomalia, D. A.; Christensen, J. B.; Boas, U. *Dendrimers, dendrons and dendritic polymers*, Cambridge University Press, **2012**.
(2) Caminade, A.-M.; Turrin, C.-O. *J. Mater. Chem. B* **2014**, *2*, 4055–4066.
(3) Carlmark, A.; Malmström, E.; Malkoch, M. *Chem. Soc. Rev.* **2013**, *42*, 5858–5879.
(4) García-Gallego, S.; Nyström, A.; Malkoch, M. *Prog. Polym. Sci.* **2015**, DOI 10.1016/j.progpolymsci.2015.04.006.
(5) Feliu, N.; Walter, M. V.; Montañez, M. I.; Kunzmann, A.; Hult, A.; Nyström, A.; Malkoch, M.; Fadeel, B. *Biomaterials* **2012**, *33*, 1970–1981.
(6) Parrott, M. C.; Benhabbour, S. R.; Saab, C.; Lemon, J. A.; Parker, S.; Valliant, J. F.; Adronov, A. *J. Am. Chem. Soc.* **2009**, *131*, 2906–2916.
(7) Zeng, X.; Zhang, Y.; Wu, Z.; Lundberg, P.; Malkoch, M.; Nyström, A. M. *J. Polym. Sci., Part A: Polym. Chem.* **2012**, *50*, 280–288.
(8) Wu, Z.; Zeng, X.; Zhang, Y.; Feliu, N.; Lundberg, P.; Fadeel, B.; Malkoch, M.; Nyström, A. M. *J. Polym. Sci., Part A: Polym. Chem.* **2012**, *50*, 217–226.
(9) Morgan, M. T.; Carnahan, M. A.; Immoos, C. E.; Ribeiro, A. A.; Finkelstein, S.; Lee, S. J.; Grinstaff, M. W. *J. Am. Chem. Soc.* **2003**, *125*, 15485–15489.
(10) Morgan, M. T.; Nakanishi, Y.; Kroll, D. J.; Griset, A. P.; Carnahan, M. A.; Wathier, M.; Oberlies, N. H.; Manikumar, G.; Wani, M. C.; Grinstaff, M. W. *Cancer Res.* **2006**, *66*, 11913–11921.
(11) Ooya, T.; Lee, J.; Park, K. *J. Control. Release* **2003**, *93*, 121–127.
(12) Mahltig, B.; Cheval, N.; Astachov, V.; Malkoch, M.; Montañez, M. I.; Haase, H.; Fahmi, A. *Colloid. Polym. Sci.* **2012**, *290*, 1413–1421.
(13) Gillies, E. R.; Fréchet, J. M. J. *Bioconjugate Chem.* **2005**, *16*, 361–368.
(14) Hed, Y.; Zhang, Y.; Andrén, O. C. J.; Zeng, X.; Nyström, A. M.; Malkoch, M. *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 3992–3996.
(15) Lundberg, P.; Walter, M. V.; Montañez, M. I.; Hult, D.; Hult, A.; Nyström, A.; Malkoch, M. *Polym. Chem.* **2011**, *2*, 394–402.
(16) Porsch, C.; Zhang, Y.; Östlund, Å.; Damberg, P.; Ducani, C.; Malmström, E.; Nyström, A. M. *Part. Part. Syst. Charact.* **2013**, *30*, 381–390.
(17) Lee, C. C.; Gillies, E. R.; Fox, M. E.; Guillaudeu, S. J.; Fréchet, J. M. J.; Dy, E. E.; Szoka, F. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 16649–16654.
(18) Lo Conte, M.; Robb, M. J.; Hed, Y.; Marra, A.; Malkoch, M.; Hawker, C. J.; Dondoni, A. *J. Polym. Sci., Part A: Polym. Chem.* **2011**, *49*, 4468–4475.
(19) Ghirardello, M.; Öberg, K.; Staderini, S.; Renaudet, O.; Berthet, N.; Dumy, P.; Hed, Y.; Marra, A.; Malkoch, M.; Dondoni, A. *J. Polym. Sci., Part A: Polym. Chem.* **2014**, *52*, 2422–2433.
(20) Montañez, M. I.; Hed, Y.; Utsel, S.; Ropponen, J.; Malmström, E.; Wagberg, L.; Hult, A.; Malkoch, M. *Biomacromolecules* **2011**, *12*, 2114–2125.
(21) Wu, P.; Malkoch, M.; Hunt, J. N.; Vestberg, R.; Kaltgrad, E.; Finn, M. G.; Fokin, V. V.; Sharpless, K. B.; Hawker, C. J. *Chem. Commun.* **2005**, 5775–5777.
(22) García-Gallego, S.; Hult, D.; Olsson, J. V.; Malkoch, M. *Angew. Chem. Int. Ed.* **2015**, *54*, 2416–2419.
(23) Mongkhontreerat, S.; Andrén, O. C. J.; Boujemaoui, A.; Malkoch, M. *J. Polym. Sci. A Polym. Chem.* **2015**, DOI 10.1002/pola.27750.
(24) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.
(25) Antoni, P.; Robb, M. J.; Campos, L.; Montañez, M.; Hult, A.; Malmström, E.; Malkoch, M.; Hawker, C. J. *Macromolecules (Washington, DC, U.S.)* **2010**, *43*, 6625–6631.

Dendrimers

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bis-MPA Dendrimers, TMP Core

Surface Group	Generation	No. of Surface Groups	Prod. No.
Hydroxyl	1	6	805920-250MG
	2	12	805939-250MG
	3	24	805947-250MG
	4	48	805955-250MG
Carboxyl	1	6	806099-250MG
	2	12	806064-250MG
	3	24	806080-250MG
	4	48	806072-100MG
Acetylene	1	6	806145-250MG
	3	24	806153-250MG
	5	96	806161-100MG

Hyperbranched PEG OH Dendrimers

Name	Pseudo Generation	No. of Surface Groups	Prod. No.
G4-PEG6k-OH	4	32	806218-5G
G5-PEG6k-OH	5	64	806226-5G
G6-PEG6k-OH	6	128	806234-5G
G2-PEG10k-OH	2	8	806242-5G
G3-PEG10k-OH	3	16	806250-5G
G4-PEG10k-OH	4	32	806269-5G
G5-PEG10k-OH	5	64	806277-5G
G6--PEG10k-OH	6	128	806285-5G
G2-PEG20k-OH	2	8	806293-5G
G3-PEG20k-OH	3	16	806307-5G
G4-PEG20k-OH	4	32	806323-5G
G5-PEG20k-OH	5	64	806315-5G
G6-PEG20k-OH	6	128	806331-5G

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Introduction

Advances in genomics and proteomics have delivered an amazing collection of new biological information and new therapeutic targets for the biopharmaceutical industry. Biologic drugs based on proteins, RNA, DNA, and cells have the broad target range necessary to translate the potential of the genome era into effective therapeutics and individualized medicines. Biologic drugs currently comprise approximately 20–30% of clinically utilized drugs, and they represent the fastest growing segment since they address needs for safety, specificity and individualized medicine. The biologics category is dominated by monoclonal antibodies for the treatment of oncology and inflammatory diseases and by vaccines for the treatment of infectious diseases. However, the number of nucleic acid and protein therapeutic biologics is still limited, and their larger potential impact still remains unrealized due to limitations in drug delivery. The crossing of biological barriers at the circulatory, tissue, and cellular level remains the key to ultimate efficacy and one that, in broad terms, continues to stymie the field of biologic drug delivery.

Pathogens display the highest level of success now known for the delivery of DNA, RNA, and proteins. Viruses and pathogenic organisms such as *Diphtheria* have evolved highly effective systems for delivery of nucleic acids and proteins to intracellular locations and targets.[1] These organisms feature remarkable molecular machines that enhance transport of DNA, RNA, or proteins across the endosomal membrane. The key functional property of these proteins is a membrane-destabilizing activity that is closely coupled to a pH-sensing mechanism. At physiological pH (7.4), the proteins are in a "stealth" conformation until they are brought into the intracellular endosomes via receptor-mediated endocytosis. As the pH of these compartments drops during endosomal development to values of 5.5 or lower, a conformational change is triggered to expose a membrane-destabilizing domain in viral proteins such as hemagluttinin, or pathogenic proteins such as diphtheria toxin.[1]

We have developed polymeric carriers based on this biological design, incorporating pH-sensing moieties that trigger membrane destabilizing activity at a defined lower pH value typical of endosomes.[2–7] These polymers are prepared using the Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization technique.[8–10] Initial designs were directed toward gene (plasmid), antisense oligonucleotide, and RNA delivery. A representative design exploited a polyampholyte segment composed of dimethylaminoethyl methacrylate (DMAEMA), propylacrylic acid (PAA), and a hydrophobizing monomer butyl methacrylate (BMA).[7] The pH-responsive DMAEMA and PAA were incorporated at equimolar ratios, leading to a charge-neutralized and hydrophobized block segment. At neutral pH, this block is core-forming when combined with a hydrophilic block segment, but it transitions into the endosomal pH range as the DMAEMA is protonated to become more positively charged and the PAA is protonated to neutrality. The corresponding micelle destabilization leads to exposure of the core segments and the carriers become sharply membrane-destabilizing at endosomal pH values. We characterized the structure–activity relationships that connected intrinsic membrane-destabilizing activity with siRNA delivery activity. A series of seven diblock copolymers were synthesized where the BMA hydrophobizing content was varied from 0–50% in the second block. It was shown that as BMA content increased, while DMAEMA and PAA were kept at equimolar but decreasing mole fraction, membrane-destabilizing activity and gene and protein knockdown increased (**Figures 1A–B**). This work provided initial confirmation of carrier activity and guided further development of active compositional designs.[4–6]

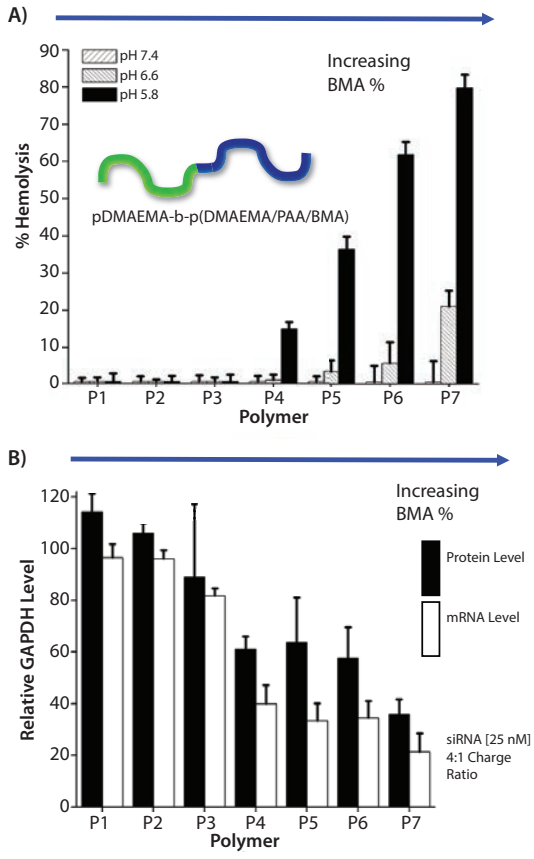


Figure 1. **A**) Structure–activity of diblock carrier series (pDMAEMA-b-(DMAEMA/PAA/BMA) in pH-dependent, membrane-destabilization hemolysis assay. P1 (0% BMA), P2 (1% BMA), P3 (12% BMA), P4 (19% BMA), P5 (24% BMA), P6 (27% BMA), and P7 (48% BMA). **B**) Structure-activity of carrier series in siRNA delivery and GAPDH knockdown assay (HeLa cells).

Antibody-drug Conjugates for Intracellular Delivery of Proteins and Peptides

Recently, we have adapted these carrier designs to the challenge of intracellular protein and peptide delivery through an antibody drug conjugate (ADC) approach.[11,12] The introduction of antibody and antibody-drug conjugate (ADC) therapeutics has had a major impact in the cancer field, demonstrating that anti-tumor efficacy can be enhanced and toxicity diminished by increasing tumor delivery selectivity.[13,14] Current ADCs exploit small molecule drugs. These small molecule drugs are typically hydrophobic enough to transport broadly inside cells to reach their intracellular targets. Additional selectivity and new therapeutic approaches could be realized by opening antibody-targeted drugs to new classes of biologic drugs that more specifically target the proteins dysregulated or abnormally produced in transformed tumor cells. The driving goal of developing new intracellular polymeric carriers is to develop ADC-like delivery systems for biologic drugs—specifically, proteins/peptides that work against intracellular targets. These protein drugs could greatly expand the repertoire of disease-specific targets that are currently undruggable with small molecules.

RAFT polymer synthesis allows for the development of well-tuned heterotelechelic diblock copolymers with endosomal-releasing capabilities (**Figure 2**). RAFT represents one of the most significant recent advances in synthetic chemistry, and its application is revolutionizing a broad range of disciplines from traditional polymer science to biology. The versatility of RAFT lies in the elegant simplicity of the technique, broad chemical compatibility, and ease of use. This technique was first reported by Rizzardo et al. at the Commonwealth Scientific and Industrial Research Organization.[8] Since that time, RAFT has become one of the premier methods to prepare sophisticated polymers for biotechnology applications. RAFT employs a thiocarbonyl thio compound as a degenerate chain transfer agent (CTA), which is most commonly a dithioester or trithiocarbonate. By simple manipulation of the initial monomer, CTA, and radical initiator stoichiometry, it is possible to prepare low dispersity materials over a range of predefined molecular weights. Because this methodology does not require the use of any toxic metal catalysts, it is particularly well-suited for use in biotechnology applications. Most typical commercially available RAFT CTAs, which contain carboxylic acid functionality, are modified using standard esterification/amidation reactions. Because the functional groups are introduced as part of the polymerization process, this strategy eliminates the need for costly and often ill-defined post-polymerization modification reactions. Following polymerization of a given monomer or monomers, the resultant macroCTAs can be isolated for use in subsequent block (co) polymerization steps. RAFT polymerizations are also well-controlled in water, assuming appropriate pH conditions are maintained, making them ideal for many bioconjugation applications.[9]

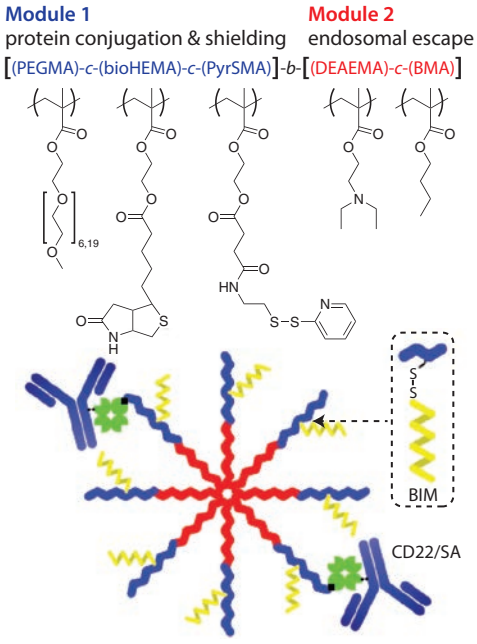


Figure 2. CD22-targeted, pH-responsive micelles loaded with proapoptotic BIM peptide. Multifunctional diblock copolymers were synthesized by RAFT polymerization with two modules. The first module is composed of PEGMA for steric shielding, bioHEMA for complexing anti-CD22/streptavidin (CD22/SA), and PyrSMA for conjugating thiol-containing BIM peptides. The second module is a block composed of DEAEMA and BMA to drive micelle assembly, endosomal escape, and cytosolic release of BIM peptides.

Previous work has demonstrated the copolymerization of diethylaminoethyl methacrylate (DEAEMA) with butylmethacrylate (BMA) by RAFT can be tuned for endosomal escape and intracellular delivery of plasmid DNA.[3] A polyethylene glycol methacrylate (PEGMA) monomer can be incorporated to add steric stabilization with "stealth-like" properties, increasing blood circulation of peptides and proteins.[15–17] The incorporation of biotinylated monomers in the corona-forming block segment facilitates the integration of antibody targeting agents and a pyridyl disulfide containing monomer provides reversible peptide/protein conjugation sites into

the carrier systems. Thus, the final hydrophilic first block is composed of three different monomers: polyethylene glycol methacrylate, pyridyldisulfide methacrylate (PyrSMA), and biotinhydroxyethyl methacrylate (bioHEMA).

In two recent papers, we demonstrated this generalized carrier system can be tailored for use with both peptide and protein therapeutics. Two segment lengths of PEG 300 and PEG 950[18] were studied and the longer-chain PEG-methacrylate monomer was found to show better PK and tumor biodistribution properties. A proapoptotic BIM peptide[19] was delivered via anti-CD22 monoclonal antibody targeting of human B-cell lymphoma in a xenograft tumor model.[11] A related carrier system was also developed for a *de novo* protein therapeutic that attacks an Epstein–Barr (EB) viral target in an EB-human B-cell lymphoma xenograft tumor model.[12] This carrier exploited anti-CD19 monoclonal antibody targeting, demonstrating the modular nature of the carrier design. Both *in vitro* and *in vivo* studies showed the carrier system could deliver active peptide drug and protein drug to tumor cells from an infusion route of administration, enabling their therapeutic activities and survival benefits.

Methods: Diblock Copolymer Biologic Drug Carriers

Synthesis of PyrSMA Monomer

In a 500 mL round-bottom flask, mono-2-(methacryloxy)ethyl succinate)(SMA) (8.9 g, 0.0387 mol, **Aldrich Prod. No.** 454974) is dissolved in chloroform (300 mL). To this solution *N*-hydroxysuccinimide (NHS), (4.89 g, 0.0425 mol, **Aldrich Prod. No.** 130672) is added and stirred for 30 min under N_2. This solution is cooled to 0 °C, and the reaction mixture is further stirred for 30 min.

N,N'-dicyclohexyl carbodimide (DCC), (9.57 g, 0.0464 mol, **Aldrich Prod. No.** 379115) and a catalytic amount of 4-(dimethylamino)pyridine (66 mg, **Aldrich Prod. No.** 107700) are added, and the solution is stirred for 1 h at 0 °C. Reaction is allowed to continue for 22 h at room temperature under N_2. After the reaction is complete, precipitated dicyclohexylurea is filtered twice.

A solution of 2-(pyridyldithiol)-ethylamine hydrochloride (1.0 g, 0.0045 mol) and Et3N (1.13 g, 0.0112 mol, **Sigma-Aldrich Prod. No.** T0886) is cooled to 0 °C and stirred for 30 min.

The previously synthesized NHS-activated SMA solution (2.2 g, 0.0067 mol) in chloroform (65 mL) is then added dropwise for 1 h. The reaction mixture is stirred overnight (16 h) at room temperature and then transferred to a separating funnel and washed with H_2O (3 × 150 mL). Organic extracts are then washed with brine and dried over Na_2SO_4, filtered, and concentrated *in vacuo* to afford a crude oil which is purified by column chromatography [SiO_2, EtOAc/hexane 3:1] to obtain 1.62 g pure product (yield=60.5%).

Example

500 mhz ^{1}H NMR in acetone D_6: δ ppm 5.81 (C**H**$_2$CCH$_3$ trans, s, 1H), 6.11 (C**H**$_2$CCH$_3$ cis, s, 1H), 1.92 (CCC**H**$_3$, s, 3H), 4.35 (OC**H**$_2$C**H**$_2$O), 3.50 (NHC**H**$_2$, t, 2H), 2.99 (NHCH$_2$C**H**$_2$, t, 2H), 2.50 (OCH$_2$C**H**$_2$COCH$_2$, t, 2H), 2.61 (OC**H**$_2$CH$_2$COCH$_2$, t, 2H), 8.48 (NCH, d, 1H), 7.81 (NCHC**H**C**H**, m, 2H), 7.47 (N**H**, b, 1H), 7.23 (CCH, d, 1H).

Synthesis of bioHEMA Monomer

Biotin (2.0 g, 8.19 mmol, 1 eqv., **Sigma-Aldrich Prod. No.** B4501) and 20 mL DMSO are added to a 50 mL round bottom flask. The biotin is then allowed to dissolve overnight in the dark.

DMAP (4.0 g, 33.7 mmol, 4 eqv., **Aldrich Prod. No.** 107700) and HEMA (4.2 g, 33.7 mmol, 4 eqv, **Aldrich Prod. No.** 128635) are added to this solution.

After the DMAP completely dissolves, DIC (5.07 mL, 33.7 mmol, 4 eqv.) is added. The solution is then septa-sealed and allowed to react for 18 h in the dark. At this time, the solution is filtered and then precipitated (1 to 20) into cold (3 °C) 150 mM HEPES buffer pH 8.4. The filtrate is then washed thoroughly with deionized water and dried under high vacuum.

Example

500 mHz ^{1}H NMR in DMSO D_6: δ ppm 1.25–1.68 (SCHC**H**$_2$C**H**$_2$C**H**$_2$, m, 6H), 1.88 (C**H**$_3$, s, 3H), 2.31 (C**H**$_2$CO$_2$, t, 2H), 2.55 (SC**H**$_2$, d, 1H), 2.82 (SC**H**$_2$, dd, 1H), 3.08 (CHC**H**(CH$_2$)S, m, 1H), 4.13 (C**H**CH(CH$_2$)S, m, 1H), 4.29 (OC**H**$_2$C**H**$_2$O and NHC(**H**)CH(CH$_2$)S, s/m, 5H), 5.7 (C**H**$_2$CCH$_3$ trans, s, 1H), 6.03 (C**H**$_2$CCH$_3$ cis, s, 1H), 6.35 and 6.43 (CON**H**CH, s, 1H).

RAFT Synthesis of macroCTAs

Two copolymers are synthesized via RAFT using Azobis(4-cyano-pentanoic acid) (ABCVA) (**Aldrich Prod. No.** 118168) as the initiator, 4-cyanopentanoic acid dithiobenzoate (CTP) (**Aldrich Prod. No.** 722995) as the chain transfer agent (CTA), with 20 wt% monomer in dimethysulfoxide (**Scheme 1A**).

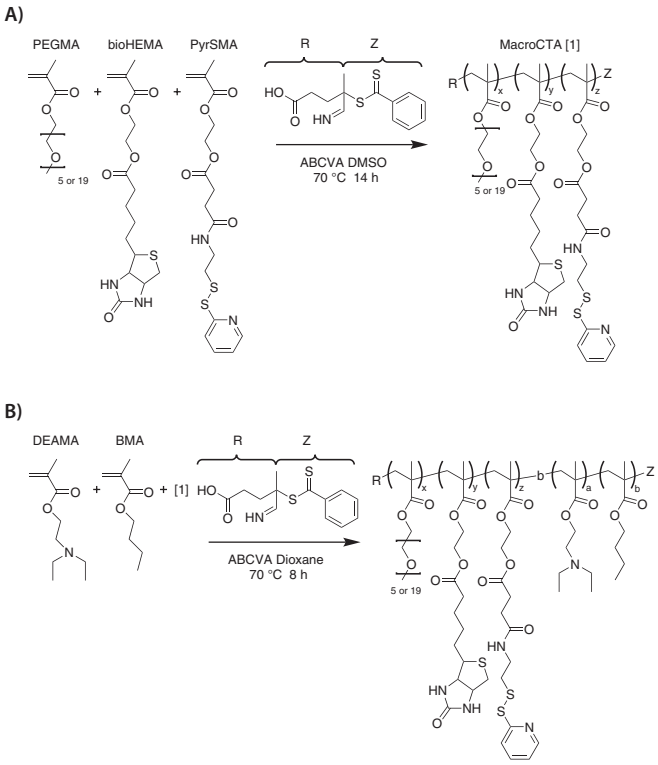
The initial monomer ($[M]_o$) to CTA ($[CTA]_o$) to initiator ($[I]_o$) ratios for the two polymers is 25:1:0.1. One reaction solution contained a feed ratio of 80% polyethylene glycol methacrylate 950 Da, 10% bioHEMA, and 10% PyrSMA. The second reaction solution contained a feed ratio of 85% polyethylene glycol methacrylate 300 Da, 5% bioHEMA, and 10% PyrSMA (**Scheme 1A**).

Individual polymerization solutions are vortexed in 20 mL scintillation vials, transferred to septa-sealed 10 mL round bottom flasks, purged under N_2 for 30 min, and transferred to a preheated water bath at 70 °C for 14 h. The resultant polymers are isolated by precipitation in diethyl ether.

The precipitated polymers are then dissolved in acetone and re-precipitated into diethyl ether (×6). The dried polymers are analyzed by ^{1}H NMR to assess purity. Gel-permeation chromatography (GPC) can be used to determine number average (M_n) molecular weight and polydispersity (PDI) of the polymers.

Example GPC Protocol

Use Tosoh SEC TSK GEL α-3000 and α-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a 1200 Series liquid chromatography system (Agilent, Santa Clara, CA) and a miniDAWN TREOS three-angle light scattering instrument with an Optilab TrEX refractive index detector (Wyatt Technology, Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt% LiBr at 60 °C is used as the mobile phase at a flow rate of 1 mL/min. Reverse-phase high performance liquid chromatography (RP-HPLC) can be used to measure monomer conversions for each macroCTA using aliquots collected at T_o and T_x.



Scheme 1. **A**)The diblock copolymer poly[(PEGMA-PyrSMA-bioHEMA)-(DEAEMABMA)] (O950) was synthesized by RAFT with a multifunctional hydrophilic first block containing PEGMA950 for *in vivo* biocompatibility, a biotin-containing monomer (bioHEMA) for antibody/streptavidin complexation, and a PDS-containing monomer (PyrSMA) for disulfide conjugation to a cysteine containing peptide. **B**) The second block contains the DEAEMA monomer which undergoes a pH-dependent phase transition and the BMA monomer for hydrophobic interactions with membrane disruption.

RAFT Synthesis of Diblock Copolymers

The two polymers isolated from the previous synthesis are used as the macroCTAs for RAFT copolymerization with ABCVA (**Aldrich Prod. No.** 118168) as the initiator in 50 wt% monomer in 1,4-dioxane (**Scheme 1B**). The [M]o:[mCTA]o:[I]o ratios for the polymers containing PEGMA 950 Da (**Aldrich Prod. No.** 447951) and PEGMA 300 Da (**Aldrich Prod. No.** 447935) are 200:1:0.2 and 125:1:0.2, respectively.

The polymer solutions contain a relative feed of 60% diethylaminoethyl methacrylate (DEAEMA, **Aldrich Prod. No.** 234907) and 40% butyl methacrylate (BMA, **Aldrich Prod. No.** 235865).

Individual polymerization solutions are vortexed in 20 mL scintillation vials, transferred to septa-sealed 5 mL round bottom flasks, purged under N_2 for 20 min, and transferred to a preheated water bath at 70 °C for 8 h. The resultant polymers can be isolated by precipitation in petroleum ether.

The precipitated polymers are then dissolved in acetone and re-precipitated into petroleum ether (×5). The dried diblock copolymers are analyzed by ^{1}H NMR, GPC, and RP-HPLC as described above and in recent publications.

Formulation of Antibody-Polymer-Peptide Conjugates

Polymeric micelles are conjugated to peptides via disulfide exchange.

First, polymers are dissolved as unimers in ethanol (100 mg/mL), then diluted 10× into DPBS, pH 7.4 to spontaneously form micelles. Ethanol content is reduced to less than 0.01% using Amicon-4 Ultra 3k MWCO spin columns (**Aldrich Prod. No.** Z740186) and the polymer concentration is verified by absorbance at 290 ηm.

The number of pyridyl disulfde (PDS) groups per polymer chain is quantified by reducing PDS groups on the polymeric micelles with an excess of Bond Breaker TCEP solution (Pierce). Conversion of PDS to 2-mercaptopyridine results in a colorimetric change that is quantified by a 343 nm absorbance with an extinction coefficient of 8,080 $M^{-1}cm^{-1}$.

Next, BIM or BIM/LE peptide is dissolved in anhydrous dimethyl-sulfoxide (30 mM, **Sigma-Aldrich Prod. No.** 276855), added to the polymer solutions and mixed vigorously by vortexing, followed by an overnight reaction at room temperature. In the final reaction mixture, the polymer concentration is 500 µM with a 1.25 molar excess of peptide, which results in one peptide bound per polymer chain. Reduction of the disulfide bond in the presence of glutathione can be confirmed by HPLC purification.

The formation of micelles and the number average diameter before and after peptide loading can be measured by dynamic light scattering following filtration through a 0.45 µm syringe filter with a polymer concentration of 0.5 mg/mL. The ability of the biotin-containing polymer micelles to complex with streptavidin and antibody/streptavidin conjugates can be quantified using a HABA assay.

Summary

These RAFT polymeric carriers provide a straightforward route to making functionally sophisticated drug delivery systems. In addition to intracellular delivery activities, additional functionalities can be synthetically incorporated. This includes the incorporation of polyethylene glycol (PEG) or alternative chemistries to optimize circulatory and biodistribution properties. Drug conjugation and targeting ligand chemistries can be incorporated via functionalized monomers or through functionalized chain transfer agents that are built into the polymer ends. Finally, the compositions can be optimized to solubilize drugs and protect more fragile drugs from enzymatic degradation in the blood and other tissues.

References

(1) Hughson, F. M. *Curr. Biol.* **1995**, *5*, 265.
(2) Bulmus, V., et al. *J. Controlled Release* **2003**, *93*,105.
(3) Manganiello, M. J. et al. *Biomaterials* **2012**, *33*, 2301–2309.
(4) Duvall, C. L. et al. *Molecular Pharmaceutics* **2010**, *7*, 468.
(5) Palanca-Wessels, M. C. et al. *Molecular Therapy* **2011**, *19*, 1529.
(6) Wilson, J. T. et al. *ACS Nano 2013*.
(7) Convertine, A. J. et al. *J. Controlled Release* **2009**, *133*, 221.
(8) Chiefari, J. et al. *Macromolecules* **1998**, *31*, 16, 5559.
(9) Lowe, A. B.; McCormick, C. L. *Australian Journal of Chemistry* **2002**, *55*, 367.
(10) Boyer, C. et al. *Chemical Reviews* **2009**, *109*, 5402.
(11) Berguig, G. et al. *Cell* **2014**, *157*, 1644.
(12) Berguig, G. et al. *Molecular Therapy* **2015**, *23*, 907.
(13) Polson, A. G. et al. *Cancer Research* **2009**, *69*, 2358–2364.
(14) Senter, P. D. *Current Opinion in Chemical Biology* **2009**, *13*, 235.
(15) Brown, A. A. et al. *European Polymer Journal* **2005**, *41*, 1757.
(16) Otsuka, H. et al. *Advanced Drug Delivery Reviews* **2012**, *64*, 246.
(17) Little, S. R.; Kohane, D. S. *Journal of Materials Chemistry* **2008**, *18*, 832.
(18) Roy, D. et al. *Polym. Chem.* **2014**, *5*, 1791.
(19) Guillaume, L. et al. *Nature Reviews Drug Discovery* **2008**, *7*, 989.

PEG MacroCTAs

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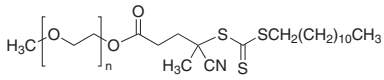
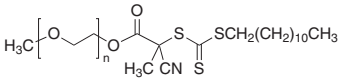
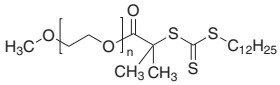
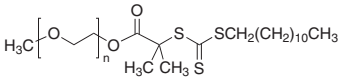
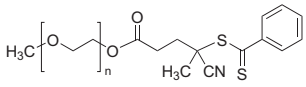
Name	Structure	Molecular Weight	PDI	Prod. No.
Poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate)		average M_n 1,400	≤ 1.1 PDI	752487-1G 752487-5G
		average M_n 2,400	≤ 1.1 PDI	751634-1G 751634-5G
		average M_n 5,400	≤ 1.1 PDI	751626-1G 751626-5G
Poly(ethylene glycol) methyl ether 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoate		average M_n 10,000	≤ 1.1 PDI	753033-1G
Poly(ethylene glycol) methyl ether 2-(dodecylthiocarbonothioylthio)-2-methylpropionate		average M_n 1,100	≤ 1.1 PDI	740705-1G
		average M_n 5,000	≤ 1.1 PDI	736325-1G
Poly(ethylene glycol) methyl ether (2-methyl-2-propionic acid dodecyl trithiocarbonate)		average M_n 10,000	≤ 1.1 PDI	752495-1G
Poly(ethylene glycol) 4-cyano-4-(phenylcarbonothioylthio) pentanoate		average M_n 2,000	≤ 1.1 PDI	764914-1G

Polylactide RAFT and ATRP Polymers

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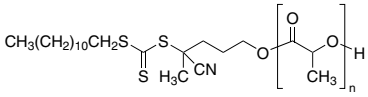
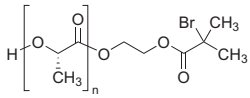
Name	Structure	Molecular Weight	Prod. No.
Poly(D,L-lactide), 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl]pentonate terminated		average M_n 5000	792020-1G
		average M_n 20,000	797790-1G
Poly(L-lactide), 2-bromoisobutyryl terminated		average M_n 3,000	773247-1G

Macro-RAFT

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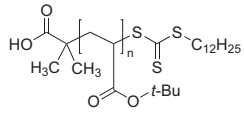
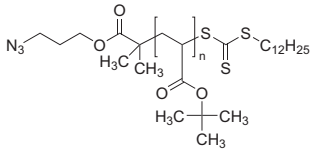
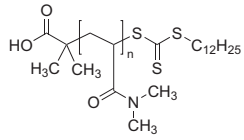
Name	Structure	Molecular Weight	PDI	Prod. No.
Poly(*tert*-butyl acrylate), DDMAT terminated		average M_n 7,000	< 1.2 PDI	772550-1G
Poly(tert-butyl acrylate), DDMAT terminated, azide terminated		average M_n 8,500	≤ 1.2 PDI	776424-1G
Poly(*N*,*N*-dimethylacrylamide), DDMAT terminated		average M_n 10,000	≤ 1.1 PDI	773638-1G

LINEAR AND BRANCHED POLYETHYLENIMINES
AS NONVIRAL VECTORS FOR GENE DELIVERY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Introduction

Gene therapy, the delivery of DNA or RNA into cells, has gained momentum over the past few decades as a potent tool for treatment of genetic disorders and cancer. A significant obstacle to gene therapy is developing effective carriers that protect the DNA or RNA from serum nuclease degradation, facilitate cellular uptake, and enable transfer of the cargo into the nucleus or cytoplasm. Nonviral vector systems, based on cationic lipids, dendrimers, peptides, and polymers,[1] are currently preferred for gene delivery because they are much safer than viral systems,[2] which are burdened by immunogenic or inflammatory responses.

Polyethylenimine (PEI) is a cationic polymer widely adopted in nonviral gene delivery systems both *in vitro* and *in vivo* due to its high transfection efficiency.[3] PEI is capable of condensing plasmid DNA and RNA through electrostatic interaction to form complexes that are internalized into cells through endocytosis. This ability is attributed to the "proton sponge" characteristic of PEI, in which the single nitrogen per monomer subunit in PEI forms an ionic interaction with the phosphate backbone of nucleic acids. Thus, DNA or RNA complexes (or polyplexes) are readily formed through mixing with PEI.[4] These PEI complexes result in increased transfection efficiency due to the large buffering capacity of PEI, leading to changes in endosomal osmolarity, and resulting in lysis and release (endosomal escape). PEI-based vectors have been used to deliver oligonucleotides, plasmid DNA, as well as RNA and intact ribozymes.[5]

The molecular weight, structure, and branching of PEI can each influence the condensation behavior, complex size, and transfection efficiency of the vector. For example, the transfection efficiency of PEI increases by increasing the molecular weight of the polymer. Higher molecular weight PEI generally results in a decreased complex size. However, higher molecular weight PEI polymers also lead to higher toxicity.[1] The cytotoxicity of PEIs is the result of the high positive charge density within the polymer chains that can lead to destabilization of the cell wall and cellular necrosis. The overall cytotoxicity of the PEI vectors can be modulated by adjusting the nitrogen-to-phosphate (N/P) ratio of the nucleotide/PEI complex.[6] The preferred molecular weight for PEI for gene delivery is estimated to be in the range of 5,000–25,000 Da, whereas lower molecular weight PEI transfection efficiency can outperform the higher molecular weight counterparts only if higher N/P ratios are used.[7]

Branched PEIs possess higher charge density and carry approximately equal amounts of primary, secondary and tertiary amino groups. Linear PEI contains only secondary amino groups and therefore linear PEIs are somewhat less cytotoxic.[8] In general, primary amines condense DNA better than other amines due to their higher protonation. In addition, binding capacity is correlated to the number of primary amines, and complex stability increases with increasing primary amine content, leading to higher transfection efficiencies. Linear PEI is a particularly effective gene transfer agent. It has lower complexation capability than branched PEI toward nucleotides, but at the same time linear PEI is more efficient than branched PEI due to its topology and lower cytotoxicity as demonstrated in a number of *in vivo* studies.[9]

The design of the PEI polymeric carrier can be challenging since transfection efficiency and cytotoxicity depend on the physiochemcial properties of the polymer. In addition, PEI can be used for different gene therapy applications as a carrier for plasmids, oligonucleotides, or siRNA. In general, molecular weight and branching need to be adjusted depending on application to design stable complexes with the desired release rates.

Methods: PEI Solutions and Complexes

Linear PEI

Linear PEI is not directly soluble in water at ambient temperature. To make a PEI solution based on monomer units (7.5×10^{-3} M), linear PEI (0.323 g/L) is added to endotoxin-free de-ionized H_2O and stirred for approximately 1 h on low heat until all of the particles are in solution. The volume is then adjusted with de-ionized H_2O to achieve the desired concentration. Allow the solution to cool to room temperature, neutralize to the desired pH, and sterile filter (0.22 µM). A large PEI stock solution can be prepared and stored frozen at –20 °C. Aliquots of linear PEI can be thawed and stored at 4 °C while in use. Aliquots of linear PEI may be adjusted to pH 7.0, 8.0, and 9.0 for testing the effect of the pH of the PEI solution on the transfection.[10]

Branched PEI Solutions

Branched PEI is an extremely viscous liquid and cannot be pipetted; therefore, stock solutions of branched PEI should be made in water, first at high concentrations (100 g/L). Once the solution is homogeneous, it is further diluted to 1 mg/mL, the pH is adjusted to 7 with HCl, then filter sterilized (0.22 µm filter) and aliquoted. Aliquots are stored frozen at –20 °C, and working solutions can be kept at 4 °C for long periods of time (months) without any loss in transfection efficiency.[11]

Formation of PEI/Polynucleotide Complexes

The size of the complexes depends on the PEI-to-DNA ratio, often referred to as the N/P ratio. The dimensions of the polyplexes can range from 50 to >1,000 nm depending on protocol conditions (i.e., N/P ratios) and whether the PEI is branched or linear.[12,13] In general, N/P ratios of 2:1 to 20:1 are used to achieve stable complexes using linear or branched PEI. Note the sequence of addition (adding PEI to DNA) can influence polyplex size and transfection efficiency.

1. Allow stock reagents to warm to room temperature.
2. Ensure cells for transfection are at the desired confluency (70–90%) to improve transfection efficiency. Replace growth medium with low serum media (<2% serum).
3. Prepare diluted PEI and plasmid separately to the desired concentrations (in endotoxin-free water, 150 mM saline, or phenol-free growth medium), vortex and leave at room temperature for 10–15 min.

   For a standard 15 cm tissue culture dish (approximate surface area of 176 cm^2), 50 µg of plasmid DNA are required (quantities need to be scaled accordingly for the different surface areas of plates/flasks/bottles). The DNA is added to 5 mL serum-free medium, and mixing is followed by the addition of 75 µL PEI stock (1 µg/mL) and brief vortexing.
4. Slowly add diluted PEI (1 µg/mL) to the DNA solution to achieve the desired N/P ratio, vortex, and incubate at room temperature for another 15 min to allow DNA/PEI complex formation.
5. Add DNA/PEI mixture to cells and briefly rotate the dish to allow mixing. Allow transfection to occur in an incubator for 3–24 h.
6. Remove transfection complexes by washing cells with PBS. Add growth medium and assay transfection efficiency.

References

(1) Mintzer, M. A.; Simanek, E. E. *Chem. Rev.* **2009**, *109*, 259–302.
(2) Karmali, P. P.; Chaudhuri, A. *Medicinal Research Reviews* **2007**, *27* (5) 696–722.
(3) Gebhart, C. L.; Kabanov, A. V. *J. Controlled Release* **2001**, *73*, 401–416.
(4) Jager, M.; Schubert, S.; Ochrimenko, S.; Fischer, D.; Schubert, U. *Chem. Soc. Rev.* **2012**, *41*, 4755–4767
(5) Aigner, A.; Fischer, D.; Merdan, T.; Brus, C.; Kissel, T.; Czubayko, F. *Gene Ther.* **2002**, *9*, 1700–1707.
(6) Erbacher, P.; Bettinger, T.; Brion, E.; Coll, J.-L.; Plank, C.; Behr, J.-P.; Remy, J.-S. *Journal of Drug Targeting* **2004**, *12* (4), 223–236.
(7) Kunath, K.; von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. *J. Controlled Release* **2003**, *89*, 113–125.
(8) von Harpe, A.; Petersen, H.; Li, Y.; Kissel, T. *J. Controlled Release* **2000**, *69*, 309–322.
(9) Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. *European Journal of Pharmaceutics and Biopharmaceutics,* **2005**, *60*, 247–266.
(10) Reed, S. E.; Staley, E. M.; Mayginnes, J. P.; Pintel, D. J.; Tullis, G. E. *Journal of Virological Methods* **2006**, *138*, 85–98.
(11) Aricescu, A. R.; Lu, W.; Jones, E. Y. *Acta Cryst.* **2006**, *D62*, 1243–1250.
(12) Goula, D.; Benoist, C.; Mantero, S.; Merlo, G.; Levi, G.; Demeneix, B. A. *Gene Ther.* **1998** *5*, 1291–1295.
(13) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. *J. Gene Med.* **2001**, 3, 362–372.

Linear PEI

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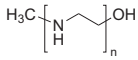
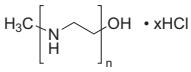
Name	Structure	Molecular Weight	PDI	Prod. No.
Polyethylenimine, linear		average M_n 2,500	< 1.2 PDI	764604-1G
		average M_n 5,000	< 1.2 PDI	764582-1G
		average M_n 10,000	≤ 1.2 PDI	765090-1G
Polyethylenimine hydrochloride		average M_n 4,000	≤ 1.1 PDI	764892-1G 764892-5G
		average M_n 8,000	≤ 1.1 PDI	764647-1G
		average M_n 20,000	≤ 1.2 PDI	764965-1G

Branched PEI

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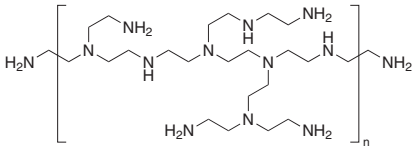
Name	Structure	Molecular Weight	Prod. No.
Polyethylenimine, ethylenediamine branched		average M_n ~600 by GPC	408719-100ML 408719-250ML 408719-1L
Polyethylenimine, branched		average M_n ~10,000 by GPC	408727-100ML 408727-250ML 408727-1L

Polyphosphoesters for Gene Delivery

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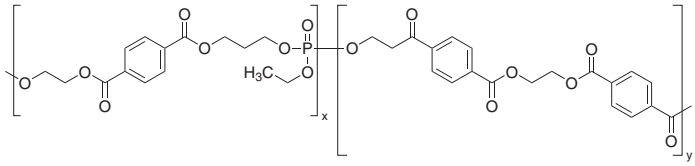
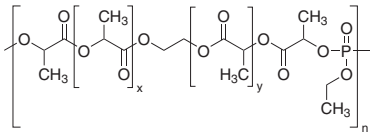
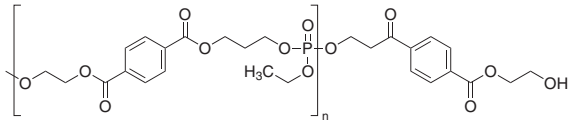
Name	Structure	Prod. No.
Poly[1,4-bis(hydroxyethyl)terephthalate-*alt*-ethyloxyphosphate]-*co*-1,4-bis(hydroxyethyl) terephthalate-*co*-terephthalate		659738-1G
Poly[(lactide-*co*-ethylene glycol)-*co*-ethyloxyphosphate]		659606-1G
Poly[1,4-bis(hydroxyethyl)terephthalate-*alt*-ethyloxyphosphate]		659614-1G

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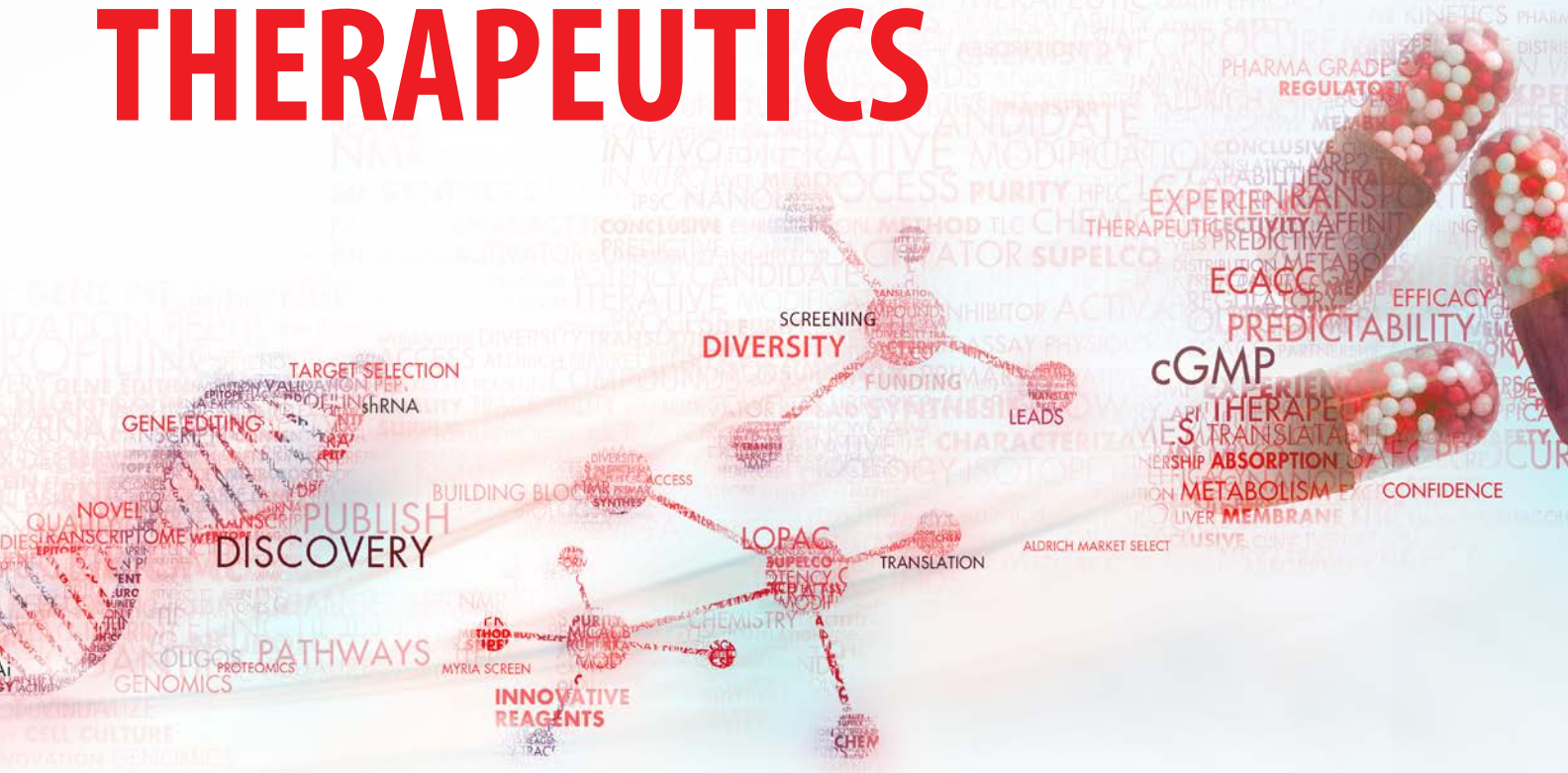

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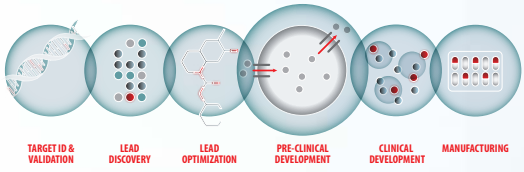

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