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Lipobeads as Future Drug Delivery System

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Introduction

In the last several decades, drug delivery systems based on encapsulation have captured great attention to address growing need in delivering the newly identified therapeutic compounds with the maximum efficacy and minimal risk of negative side effects [1-4]. Combination of polymeric and liposomal micro- and/or nanocapsules is a natural way to reach the next level of complexity of drug carrier systems. Indeed, a bilayer membrane supported by an elastic polymer network is the unique achievement of Nature in constructing multifunctional, flexible, and dynamic machineries, called cells. This chapter deals with the synthetic assemblage of liposomes and hydrogel micro- and nanoparticles. Different terminology, such as supramolecular biovectors (SMBV) [5], lipid-coated microgels [6,7], lipobeads [8-14], gel-filled vesicles [16], lipogels [17,18], gel core liposomes [19], microgel-in-liposomes [20], hydrogel supported lipid bilayer [21], nanolipogels particles (nLG) [22] has been utilized to describe these lipid membrane/hydrogel structures. Here, the term “lipobeads” is used for the bipartite structures consisting of the hydrogel core enclosed within a lipid bilayer. In this chapter, an updated view on functionality and synthetic feasibility of the lipobeads as precursors for novel encapsulated and combined drug delivery systems is presented. The chapter is meant to be a useful source of references for the researchers in the field of drug delivery design both in academia and in industry. It may be predicted that in the future, the demand in this information will rise with a growing interest in the encapsulated drug delivery systems with tiny bioscopic mechanisms of drug release.

Nanoparticulate drug delivery systems

The beginning of nanoscopic era in the development of drug delivery systems was associated with three major concepts conceived by an international team of scientists and clinicians, namely: (i) the concept of polymer-drug conjugates, in which drug molecules are covalently attached to a polymeric chain (carrier) through a biodegradable linker [23-25], (ii) the concept of active targeting [26,27], in which antibodies, peptides or cell ligands are conjugated to a polymeric carrier to deliver nanodrugs to the specified cells, and (iii) the concept of passive targeting based on the so-called “enhanced permeation and retention effect” (EPR) [28,29], i.e. the ability of nanoscale carriers to reach cancer cells due to leaky vasculature of a fast-growing tumor. There are numerous nanoparticulate drug delivery systems (nanotherapeutics) studied and developed to date. Importantly, these systems are multicomponent systems, which require additional, more complex technological steps in production as compared with small molecule drugs. Let us consider different types of nanoscopic drug carriers, firstly, subdivided in two groups (polymeric and liposomal), and secondly, systemized in order of their complexity.

Polymeric nano-therapeutics

Polymer–drug conjugates. First, polyethylene glycol or PEG has been proposed as a polymeric carrier for recombinant proteins [23,24] in order to enhance their circulation time and stability against an enzyme attack or immunogenic recognition. Another polymeric carriers, such as poly(hydroxypropyl methacrylamide or PHPMA [30,31], poly(glutamic acid) or PGA [32-36], poly (1-hydroxymethylethylene hydroxymethylformal) or PHF [37], cyclodextrin [38], carboxymethyldextran [39], and oxidized dextran [40] have been synthesized to conjugate with doxorubicin [41-43] and other small molecule anti-cancer drugs (e.g., paclitaxel [32-35,44-46], platinates [47,48], camptothecin [36-39,49,50]).

The idea of polymer-drug conjugation was put forth in the mid-1970s and since that time a number of advantages over traditional chemotherapy had been realized (for references see Ch. 3 in [4]):(i) extended water solubility of a conjugated drug, (ii) improved stability against chemical and enzymatic degradation, (iii) reduced elimination rate and prolonged circulation time in comparison with a free drug, (iv) bypassing drug resistance mechanism, and (v) extended and prolonged accumulation in the tumor tissue (EPR effect). Further development continues in the following innovative directions of delivering more than one drug (combination therapy) [51-53] and using novel polymeric carries, such as dendrimers and dendronized copolymers [54,55], hyper branched polymers [56,57] to increase circulation time and drug-loading capacity, self-immolative polymers [57-59] to trigger the release of drug molecules by the cleavage of terminal protective groups, and stimuli responsive polymers [60] to facilitate drug delivery upon either a mild biological change or an external trigger (temperature [61], pH [62,63], radiation [64,65], etc.). In Figure 4.1, polymer-drug conjugates are shown as a starting point for development of the polymeric delivery systems.

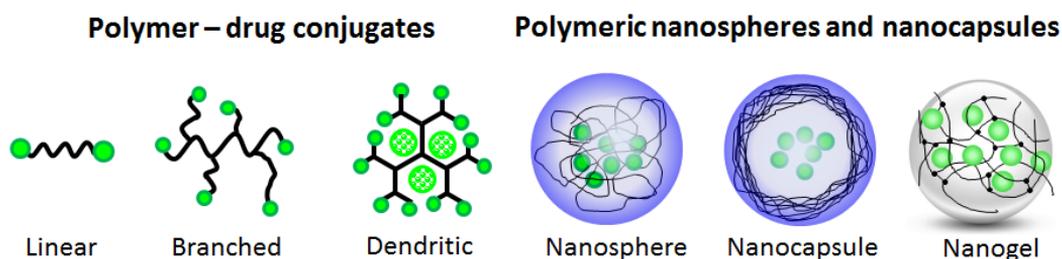


FIGURE 4.1

Representation of polymeric nanoscopic drug delivery systems in order of their complexity (part I)

Polymeric nanospheres and nanocapsules. Special class of polymeric nanoparticles is represented by nanospheres and nanocapsules, which differ in that nanosphere is solid in bulk, whereas nanocapsule consists of a central cavity (oil droplet) surrounded by polymeric membrane (Figure 4.1). Therefore, nanospheres can be loaded throughout the particle matrix, whereas in nanocapsules, the empty interior is the space for drug encapsulation. In both cases, a drug, as well as targeting molecules, can be attached to the surface of nanoparticles (not shown in Figure 4.1). Nanospheres prepared using amphiphilic block copolymers allow loading of hydrophobic drug to increase bioavailability.

The structure and properties (and even the name) of nanoparticles depend on polymer chemistry, composition, and formulation method, namely: (i) non-modified or ligand-modified nanospheres composed of a polymer or block copolymers can be prepared by the so-called nano-precipitation method [66-68], (ii) nanospheres made of dendrimers using a convergent or divergent synthesis scheme [54] are called dendritic nanoparticles [69], (iii) nanocapsules containing anticancer drug (e.g., paclitaxel) can be formed by oil-in-water interfacial polymerization [70], (iv) nanoparticles with physically or chemically cross-linked polymeric matrix fall into a special class of drug delivery systems named nanogels [71-74], (v) monodispersed polymer particles of a variety of shapes on the micro- and nanometer scale fabricated using an imprint lithography technique were named as PRINT nanoparticles (PRINT = Particle Replication in Nonwetting Templates synthesis) [75,76].

Polymeric micelles and polymersomes. Another line of polymeric carriers made of hydrophilic and hydrophobic blocks is shown in Figure 4.2. The so-called amphiphilic block conjugates tend to self-assemble into micelles or vesicles (polymersomes) in aqueous solutions. This property makes polymeric micelles and polymersomes suitable for delivery of hydrophobic drugs. The size of block

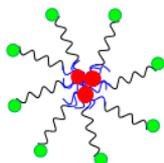
copolymer micelles ranges from 5 to 100 nm, whereas polymer vesicles vary from 40 nm to hundreds of microns in diameter.

Polymer – drug conjugates

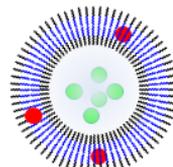


Amphiphilic block copolymer
5-100 nm

Polymeric nanospheres and nanocapsules



Micelle
5-100 nm



Polymersome
40 nm - hundreds of μm

FIGURE 4.2

Representation of polymeric nanoscopic drug delivery systems in order of their complexity (part II, block copolymer based)

In micelles, a drug is physically entrapped in a hydrophobic core or covalently bound to different moieties of the block copolymer. The earliest examples of polymeric micelles were made of PEG-hydrophobic amino acid diblock [77,78] and PEO-PPO-PEO triblock (Pluronic®) copolymers [79] by their spontaneous self-assembly into the drug-loaded PEGylated structures. Many different polymeric micelle-based systems are now under development for drug and gene delivery [80]. Polymer vesicles are spontaneously formed by a number of di/triblock copolymers [81]. Polymersomes are used for loading both water soluble drugs into the aqueous core and/or water insoluble drugs into hydrophobic membrane.

Clinical achievements of polymeric nano-therapeutics. Despite the first polymer-drug conjugate (PK1) entered clinical evaluation more than 20 years ago, and despite numerous polymeric nanoparticles are under development or undergo clinical trials, only three conjugates were approved for use so far: (i) in 2003, conjugated drug Somavert® (Pharmacia & Upjohn) was approved in the United States as a prescription medicine for treating patients with acromegaly, (ii) in 2005, nanoparticles (130 nm) composed of albumin-bound paclitaxel (Abraxane®, Abraxis Bioscience) were approved in the United States as a chemotherapeutic agent with enhanced solubility, improved circulation time and pharmacokinetics, and reduced side effects, (iii) in 2007, diblock copolymer-paclitaxel conjugated drug Genexol-PM® was approved in Korea for breast cancer treatment.

Chemically cross-linked nanogels. Among the polymeric nano-therapeutics, the highest level of complexity can be assigned to nanogels (Figure 4.3). Nanogels differ from other polymeric nanoparticles in that their structure is cross-linked to form a 3D polymer network with long chain molecules immersed in an aqueous medium. Made of natural or/and synthetic polymers, nanogels are able to absorb water up to a thousand-fold of their dry weight to contain over 99.9% water. Being cross-linked by chemical (covalent bonds) or/and physical (ionic bonds, entanglements, crystallites, charge complexes, hydrogen bonding, van der Waals or hydrophobic interactions) cross-links, the 3D hydrogel network is stabilized as a gigantic single molecule. As a result, hydrogels exhibit both liquid-like and solid-like behavior. Thermodynamically, a hydrogel is an open container with semipermeable boundaries, across which water and solute molecules can move whereas charged (ionizable) groups fixed on the network chains cannot move.

Three straightforward methods for synthesis of chemically cross-linked polymeric nanogels can be distinguished: (i) cross-linking polymer chains within already formed nanoparticles using, for

example, emulsion polymerization technique [73,82,83], (ii) polymerization within the liposomal interior followed by the lipid bilayer removal [84,85], and (iii) photolithographic fabrication of sub-micrometer hydrogel particles using the PRINT technique [75,76] or step and flash imprint lithography (S-FIL) [86] as an alternative nanoimprint photolithographic approach.

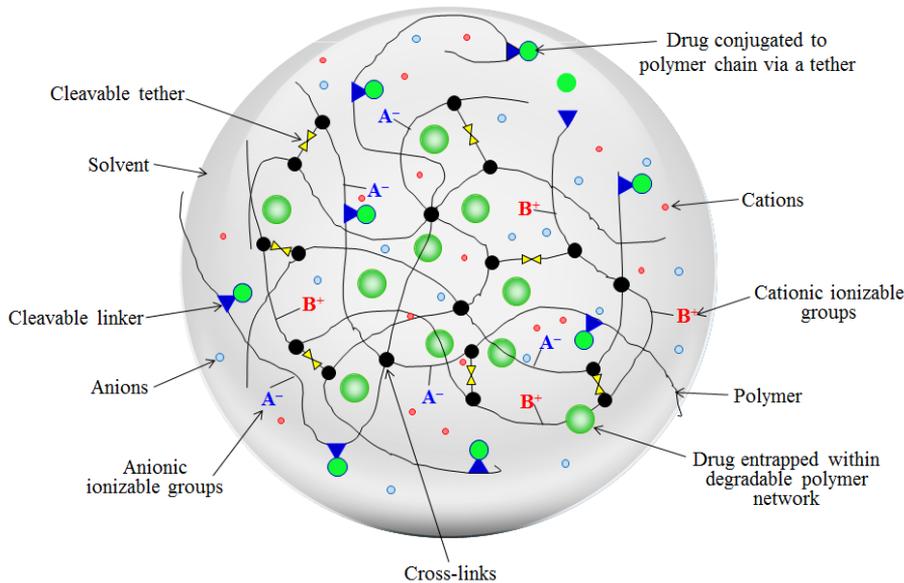


FIGURE 4.3

A futuristic view on a stimuli-responsive nanogel with entrapped or/and tethered drugs

Therefore, due to inherent cross-linking nanogels are stable mechanically, exhibit loading capacity suitable for drug delivery, and, what is the most important, are environmentally responsive. It has been well documented [71-74,87-89] that depending on the composition of a gel/solvent system, the polymer and cross-linking chemistry, nanogels swell or shrink discontinuously or continuously, reversibly or irreversibly in response to many different stimuli (temperature, pH, ion concentration, electric fields, light, reduction/oxidation, enzymatic activity, etc.). A significant magnitude (up to thousand-fold) and relatively high rate (from seconds to microseconds) of volume changes in the nanometer scale make polymeric nanogels an exciting and promising drug delivery system.

There is a number of options for drug delivery using the stimuli-responsive nanogels: (i) a drug can be conjugated to the polymer network through a cleavable tether (Figure 4.3), so that when the tether is cleaved, the drug is allowed to diffuse into the nearby medium, (ii) a drug can be trapped within either an environmentally sensitive polymer network or a network which contains environmentally responsive cleavable linkers, so that when the environmental conditions change, the network either changes its volume (swells/shrinks) or degrades, allowing the drug to be released. For example, the recently reported doxorubicin-loaded, pH- and redox-sensitive poly(oligo(ethylene glycol) methacrylates-ss-acrylic acid) nanogels exhibit strong internalization by human hepatocellular carcinoma cells (Bel7402) under reduced opsonization and phagocytosis, with the intracellular glutathione (GSH) as a trigger for release of doxorubicin from the nanogels into cytosol for subsequent entering the nucleus [90].

Liposome-encapsulated drug carriers

The concept of liposomal drug delivery system has been established [91,92] shortly after the discovery of liposomes [93]. From the very beginning, it became clear that entrapment of both hydrophilic and hydrophobic drugs into a liposomal interior (Figure 4.4) could improve drug biodistribution *in vivo* compared to other delivery systems [94-96]. However, the studies on the liposome-encapsulated drug carriers based on early “plain” (traditional) liposomes revealed a number of problems in their use *in vivo*, namely: (i) the difficulty in loading of some types of drugs, leakage of contents from the liposomal interior, and effect of serum proteins on drug release [92,96-98]; (ii) rapid clearance of liposomes from circulation by uptake into the cells of the mononuclear phagocyte system (MPS), predominantly in the liver and spleen [94,99,100]; and (iii) cellular and intracellular barrier to liposomal delivery [101].

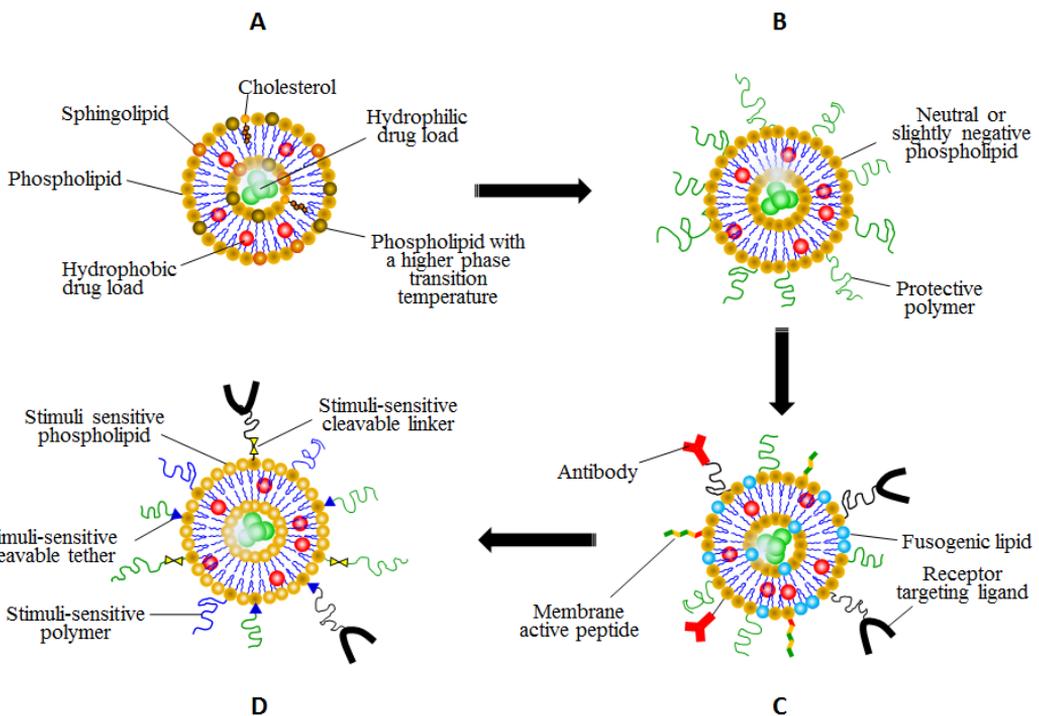


FIGURE 4.4

Evolutionary steps of liposomal drug delivery systems in order of their complexity: **A** – classical “plain” liposome with reduced leakage of drug (usage of phospholipid with the gel-to-liquid phase transition temperature higher than physiological one ($>37^{\circ}\text{C}$), incorporation of cholesterol and/or sphingolipids); **B** – “stealth” liposome with prolonged circulation (usage of neutral or slightly negative phospholipids, diameter around 100 nm, modification of liposome surface with protective polymers such as PEG); **C** – ligand-conjugated liposome targeting specific cells, intracellular organelles, tumor microenvironment and/or facilitating receptor-mediated endocytosis (attachment of antibodies, folate, transferrin, tyrosine kinase, vascular endothelial growth factor, introduction of fusogenic lipids, and membrane active peptides); **D** – stimuli-sensitive liposome with drug release controlled by external (temperature, radiation, ultrasound) or internal (pH, enzyme, redox) triggers

The primary development of liposomal drug delivery systems aimed at overcoming these obstacles. For example, to reduce leakage from liposomes, cholesterol [102] or/and sphingomyelin [103] were incorporated into lipid bilayer and phospholipid with a higher phase transition temperature [104] was used to form a more solid lipid bilayer.

Loading and retention of drugs in liposomes are drug dependent processes. Many drugs are weak bases, which can be loaded in response to pH gradients [105-109]. Some drugs, like doxorubicin, exhibit good retention properties under conditions enhancing their precipitation inside liposomes [110-112], whereas retention of highly hydrophobic drugs, like paclitaxel, in liposomes is still challenging [113,114], but they can be encapsulated and retained in liposomes if converted to the weak bases [115]. It is obvious that in order to provide reasonable therapeutic activity at the targeted site, the rate of drug entrapment should be optimized with respect to the rate of drug release [116-118]. One could distinguish two methods for triggering the release of liposomal contents at the targeted site, namely: remote triggers include heat and radiation and local triggers such as enzymes and pH changes (see review [119] and references therein). It is found that the clearance of liposomes is slower if they are neutral or slightly negative and their size is around 100 nm [100,120]. Plus, the so-called "stealth liposomes" with prolonged circulation were developed by stabilizing the liposomes with protective polymers (e.g., polyethylene glycol, PEG) [121].

There are three ways to facilitate the intracellular drug delivery: (i) introduction of fusogenic lipids or membrane active peptides into liposomal bilayer enhances fusion or even disruption of cell/organelle membrane and thereby improves cytoplasmic delivery of drug [122-126], (ii) utilization of macrophages for natural endocytosis of drug-loaded liposomes [127], and (iii) receptor-mediated endocytosis of ligand-targeted liposomal drug carriers into the intracellular compartment (see reviews [3,26,128,129]).

As shown in Figure 4.4, the evolution of liposomal delivery systems in the order of increasing complexity includes classical liposomes, stealth liposomes, ligand conjugated liposomes, and stimuli-sensitive liposomes.

Liposomes have been used as carriers for many kinds of molecules such as anti-cancer, anti-bacterial, anti-fungal and anti-viral agents, and bioactive macromolecules (see [12] for references). The liposomal drug delivery systems, which have already been approved to market and are in clinical development, are well documented in the recent reviews [3,130-135]. Table 4.1 summarizes the most clinically successful liposomal anticancer products.

TABLE 4.1

Approved liposomal anticancer products

Name	Company	Drug	Cancer	Approval	
				Country	Year
Doxil®/ Caelyx®	Janssen Pharmaceuticals	Pegylated liposomal (90 nm) doxorubicin	Kaposi sarcoma	USA	1995
			Ovarian cancer		1999
			Breast cancer		2003
LipoDOX®	Sun Pharmaceuticals, India	Generic form of Doxil	Kaposi sarcoma Ovarian cancer Breast cancer	Taiwan	2002
Myocet®	Cephaton-TEVA, USA	Non-pegylated liposomal (180 nm) doxorubicin in combination with cyclophosphamide	Metastatic breast cancer	Europe	2000
	Sopherion Therapeutics, Israel			Canada	
DaunoXome®	Galen, UK	Non-pegylated liposomal (60 nm) daunorubicin (analog of doxorubicin)	Kaposi sarcoma	Europe &USA	1996
Marqibo®	Talon Therapeutics	Vincristine sulfate liposome injection, a sphingomyelin and cholesterol-based liposomal formulation of vincristine	Acute lymphoblast ic leukemia	USA	2012
Lipusu®	Luye Pharma Group, China	Liposomal paclitaxel	Solid tumors (ovary, breast and non-small cell lung cancer)	China	2006

Lipobeads: raising the complexity level of drug carrier systems

Mimicking natural constructs

It is worthy to notice that both polymeric (Figures 4.1 and 4.2) and liposomal (Figure 4.4) drug delivery systems tend to develop in the direction of increasing complexity, *i.e.* in accord with our understanding of the complex biological mechanisms prevailing *in situ*. To this point, one can conclude that the next level of complexity is multifunctional [136] and multicompartmental [137,138] drug delivery formulations achievable experimentally in laboratory. A logical combination of polymeric and liposomal beaded nanoscopic systems is the arrangements of the lipid bilayer/hydrogel assembly – *lipobeads* – the lipid vesicles filled with polymeric networks (Figure 4.5).

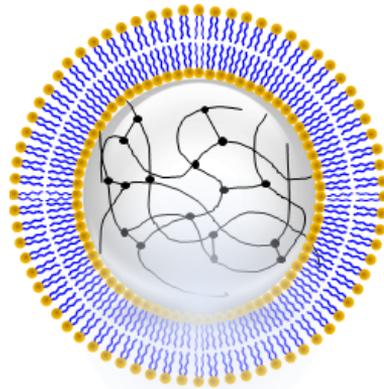


FIGURE 4.5
Schematic of the spherical lipid bilayer/hydrogel assemblies (lipobeads)

The bi-compartmental structure of lipobeads mimics natural configurations of living cells. Indeed, the sketches of cell envelopes for representatives of three main domains of life – *eubacteria*, *archaea* and *eukaryotes* (Figure 4.6) – leap to the eye as a successive organization of the macromolecular networks (cytoplasm, cell wall, capsule, etc.) and lipid bilayers (cell bilayer membranes, internal membrane system), see for example [139-141]. It looks like Nature uses a combination of properties of both lipid bilayer and cross-linked (physically or chemically) polymer network to provide workability, multifunctionality, dynamism of living cells of different types.

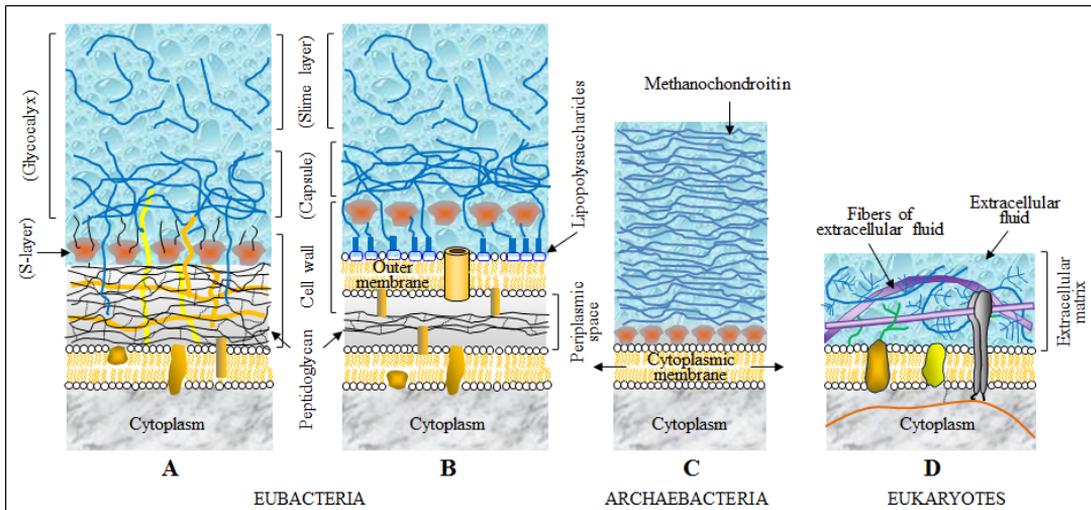


FIGURE 4.6
Examples of cell envelopes for gram-positive (A) and gram-negative (B) bacteria, archaeobacteria (C, e.g., *Methanosarcina*), and animal cell (D). Structural layers in parentheses are not found in all cells

On the one hand, lipid bilayer, being impermeable to water-soluble (hydrophilic) molecules, is ideally suited to the role of the cell membrane of almost all living organisms as well as the

membranes surrounding the sub-cellular structures. On the other hand, in naturally occurred structures – living cells – the matter enclosed within the cell membrane (cytoplasm) consists of cytosol (the gel-like substance) and organelles (the cell's internal sub-structures). It is within the cytoplasm that most cellular activities occur, such as metabolic reactions and cell division. Interestingly, the portion of the cytoplasm not contained within membrane-bound organelles (~70% of the cell volume), *cytosol*, is a complex mixture of protein filaments that make up the cytoskeleton, dissolved molecules of soluble proteins, salts, and water. Due to this network of fibers and high concentrations of dissolved macromolecules, the cytosol does act as a temperature- and ion-sensitive hydrogel. A unique property of hydrogels is the abrupt volume changes, transition from a collapsed to swollen state and backward, in response to external stimuli (cf. Figure 4.3).

Historical prospective

Evidently, the first lipid vesicles filled with hydrogel (lipobeads) were reported in 1987, when a successful polymerization within liposomes had been accomplished [142] and microspherules of agarose-gelatin filled with gold particles had been encapsulated within liposomes in the course of their preparation [143]. In 1989, a concept of supramolecular biovectors (SMBVs) was filled as a patent application [144]. The SMBV system was prepared from polysaccharide gel fragments obtained by disruption of a gel of chemically cross-linked maltodextrins and subsequently phospholipidated. In 1994, the SMBVs were reported as new carriers of active substances, such as interleukin-2 (IL-2) [145]. In 1995, lipobeads with Ca-alginate hydrogel core were obtained as a byproduct of a method for the preparation of Ca-alginate hydrogel nanoparticles using the internal compartment of liposomes [146]. In 1996, the spherical hydrogel/lipid bilayer constructs were, for the time first, named as “lipobeads”, and it was shown that a lipid bilayer was formed on the surface of hydrogel polymer beads upon the addition of phospholipids, if their surface had been modified with covalently attached fatty acids [8]. Lipobeads with an environmentally sensitive hydrogel core prepared by hydration of lipid films with microgel suspension were described as a drug delivery system in 1998 [6]. In the early 2000s, photopolymerization within liposomes was used for preparation of the so-called synthetic polymer complements with imprinted recognition sites [147] and the environmentally responsive nanogels [84]. The latter work was also a contribution towards the characterization of the compatibility of nanogels and phospholipid bilayer leading to spontaneous phospholipidation of nanogels and the behavior of lipobeads obtained upon mixing anchored or unanchored stimuli-responsive nanogels with liposomes [148]. Further studies on lipobeads development were devoted either to new methodologies including different compositions of hydrogel core or different agents which could be loaded into the lipobeads. Depending on the size, lipobeads can be classified into two groups: nanolipobeads (< 1000 nm) and giant lipobeads (> 1 μm). Nanolipobeads are the objects relevant for the development of realistic drug delivery systems. The concept of giant lipogels is very important as a model for the direct study of lipobeads' structural functionality, drug loading and release mechanisms [149] using optical microscopy.

Focus of this work

Bringing a new drug through all stages of development (discovery, clinical testing, and regulatory approval) is an expensive and time-consuming process. For example, for liposomal drug delivery systems it took almost 40 years from the concept to the established technology and clinical acceptance. The concept of the lipobeads has been proposed about 30 years ago, however, they

still are at the stage of discovery and development. Keeping in mind that a considerable growth of attention to lipobeads as a new drug delivery system is not too far in the nearest future, this chapter addresses three main issues, which should be recognized to push them to the next level of development (clinical trials). First, I review the recent approaches to establish a technological platform for the lipobeadal drug delivery, i.e. two major methods for lipobeads' synthesis [polymerization within liposomal interior (method for prevention of polymerization outside liposomes) and liposome/hydrogel mixing (microgel preparation, liposome preparation, method of mixing)]. I am also going to estimate processability of the methods based on the recently obtained data. Second, the mechanisms of controlled drug release using the lipobeads with environmentally responsive hydrogel core are discussed with the emphasis on the "thermophilic" and "thermophobic" hydrogel core (drug loading, drug release). And, third, the future of lipobeads as a recognized combination and multifunctional drug delivery system is outlined in terms of their applications.

Approaches to Lipobeads Synthesis

Strategies for lipobeads preparation

The preparation of lipobeads is determined by contradictory requirements on physicochemical properties of the lipid bilayer in the courses of fabrication, loading, delivery, and release. On the one hand, liposome should be sealed enough to retain the concentration of pre-gel components (preparation) and therapeutic agent (delivery). On the other hand, lipid bilayer should be sufficiently permeable to provide the drug flow to interior without losing the bilayer integrity during drug loading and drug flux to exterior in the course of drug release. In addition, a lipid bilayer should be stiff enough to withstand a complex environment in the bloodstream and immunological attack at the sub-organ level and elastic enough at the subcellular level to provide lipobead trafficking to cytosol and intracellular organelles (nucleus, mitochondria, etc.). Presumably, similar to liposomes, the sizes of lipobeads suitable for delivery fall into a relatively narrow interval around 100 nm. The larger particles would be limited in trespassing the capillary pores, whereas the smaller particles would be removed from circulation by the active capturing system of the liver known as reticuloendothelial system (RES) (see section "The fate of systemically administered lipobeads" of this chapter).

The methods available to date for preparation of artificial bilayer-coated hydrogel particles (lipobeads, giant or nano) can be divided into two groups: The first one (Table 4.2) uses the liposomal interior as a chemical reactor for the formation of hydrogel by polymerization [22,84,142,147,149,150,152-158,160]. The second one (Table 4.3) is based on the formation of lipid layers around hydrogels after microgel-liposome mixing. In this case, lipid bilayer adsorption on the surface of hydrogel particles prepared separately is promoted via Coulombic attraction between the charged microgels and oppositely charged lipids [6,167,169], hydration of lipid films with micro- or nanogel suspension [143,144], introduction of hydrophobic anchors at the microgel surface around which adsorbing lipids may assemble [8,168,14,15,17], centrifugation of microgels onto a lipid film [7], microfluidic flowing [158], and emulsification [19,20,151].

TABLE 4.2 Lipobeards prepared by polymerization within liposomal reactor

Ref./Year	Size	Hydrogel core composition	Lipid bilayer composition (T ₁)	Method for pre-lipobeard preparation	Prevention of macroscale gelation	Gelation mechanism	Medium (pH)	Property of hydrogel core	Load
[142]/1987	~600 nm	PAAm/MBA	EPC-10°C/cholesterol	Reverse phase evaporation/sonication	Gel permeation chromatography (GPC)	Chemical (UV)	0.15M PBS (pH7.4)	Not specified	-
[146]/1995	~0.8 µm	Alginate/Ca ²⁺	DPPC (42°C)	Lipid film hydration/extrusion	Centrifugation, filtration, GPC	Ionotropic (Ca ²⁺)	Not specified	Degradable	-
[147]/2001	0.2-1 µm	PSA/BAA	PC/cholesterol	Lipid film hydration/extrusion	GPC	Chemical (UV)	10 mM PBS (pH7.2)	Not specified	-
[84]/2002	~150 nm	Anchored and non-anchored PNIPAA-VI/MBA	EPC(-10°C)	Lipid film hydration/sonication	Dilution	Chemical (UV)	50mM Tris-HCl (pH7.5)	Temperature and pH sensitivity	-
[150]/2002	~200 nm	Anchored PNIPAA/TEGDM	EPC(-10°C)	Detergent removal or extrusion	Dialysis	Chemical (UV)	Water	Probably temperature	-
[149]/2004	~10-30 µm	Agarose/cooling	DOPC(-22°C)	Electroformation	Dilution	Thermal (cooling)	20 mM sucrose	Not specified	-
[151]/2004	~1-40 µm	K-carrageenan/cooling	Egg PC(-10°C)	Emulsification	Solvent exchanges	Thermal (cooling)	Water	Degradable	-
[152]/2005	~100 nm	PNIPAA/MBA or PAAm/MBA	EPC(-10°C)	Lipid film hydration/extrusion	Dilution	Chemical (UV)	50mM Tris-HCl or 50 mM PBS	Temperature sensitive-	Hemoglobin loading [153]
[154]/2005	~350 nm	dex-HEMA	SOPC/DOTAP (?)	Lipid film hydration/extrusion	Dilution	Chemical (UV)	50mM PB (pH7.0)	Enzymatically degradable (dextranase)	-
[155]/2005	~10-50 µm	PEDOT/PSS/Ca ²⁺	Lipid extract composition	Lipid film hydration	Microinjection	Ionotropic (Ca ²⁺)	Buffer (pH7.8)	Electrically conducting	-
[156]/2006	~100 nm	PAA/MBA	DOPC(-22°C)/EPC-10°C/cholesterol	Lipid film hydration/extrusion	Radical scavenging by ascorbic acid	Chemical (UV)	HEPES (pH7.4)	Probably pH sensitivity	=
[19]/2009	~1-2 µm	PAA-pH	EPC-10°C/cholesterol	Reverse phase evaporation	Centrifugation and dialysis	Ionotropic (H-bonds)	PBS (pH7.4)	pH-sensitivity; biodegradable	BSA (antigen model)
[157]/2009	~150-300 nm	PNIPAA/MBA	DPPE(42°C)/cholesterol/DOPC	Hydrodynamic focusing in microfluidic device	Gel filtration	Chemical (UV)	0.01 M PBS (pH7.4)	Temperature sensitivity	-
[158]/2010	~0.2-100 µm	PNIPAA/MBA	DOPC(-22°C)	Electroformation	Dilution	Chemical (UV)	0.1 M sucrose (not specified)	Temperature sensitivity	-
[160]/2011	~120 nm	PLA-PEG-PLA diacrylate	Coated DOPC(-22°C)	Lipid film hydration/extrusion	Dilution	Chemical (UV)	1X PBS (not specified)	Temperature, p <i>H</i> , p <i>i</i> sensitive	Combination of TGF-β receptor-I inhibitor and immunostimulant IL-2

TABLE 4.3 Lipobeads prepared by liposome/hydrogel beads mixing

Ref./Year	Size	Hydrogel core composition	Bead preparation	Liposome composition (I)	Liposome preparation	Medium (pH)	Property of hydrogel core	Load
[143]/1987	~1-5 μm	Agarose-gelatin MG-cooling	Emulsification	22°C) cholesterol/DOPG(-18°C)/olein	W/O emulsion, organic solvent evaporation	5% dextrose HEPES (pH7.4)	Degradable	Colloidal Au, Rxn of AuCl ₃ and tannic acid within MGs
[144, 145, 161]-164]/1989	~30-60 nm	Cross-linked polysaccharide fragments	Extrusion in high-pressure homogenizer	EPC(-10°C)/cholesterol DPPC(42°C)/cholesterol	Injection of ethanol solution of PL in water, homogenization	PBS (pH7.4)	Temperature, pH, pI: core/bilayer interactions	Interleukin -2 (IL-2, [161]), antigens, suspension and incubation
[8]/1996	1-100 μm	Acylated PVA beads/ freeze-thaw	ISP	EPC(-10°C)	Lipid film hydration/sonication	HEPES (pH7.4)	-	Ca ²⁺ , Fluorescent probe, absorptive loading
[6]/1998	~6 μm	PMMA-NPMA/MB microgels	Precipitation polymerization	DPPC(42°C)/DPPG(41°C)/cholesterol SOPC/DOPG(-18°C)	Lipid film hydration/extrusion	Tris (pH7.0)	pH	Doxorubicin, MG incubation at pH and release after electroporation
[9]/2001	~50 μm	-	-	10°C)/DMPE(50°C)/cholesterol	-	HEPES (pH7.0)	-	-
[10]/2004	~600 μm	Anchored PDMAAn /E-BIS microgels	ISP	EPC(-10°C)/PS/cholesterol	Lipid film hydration/extrusion	HEN (pH7.4)	-	Hydrophilic solutes, fluorescent probe, MG incubation
[11]/2004	~5-30 μm	-	-	DMPC(24°C)/DPPC(42°C)	-	PBS (pH7.4)	-	ATP, LB incubation at a higher temperature
[165]/2004	~100 μm	-	-	EPC(-10°C)/PS/cholesterol	Lipid film hydration/sonication	HEN (pH8.0)	Antibody-antigen interaction	Transmembrane receptors, incubation with liposomes
[166]/2004	~80-100 μm	Acylated PVA beads/ freeze-thawing	ISP	PE (63°C)/olein oil/cholesterol	Lipid film hydration/sonication	HEPES (pH7.4)	-	AHA, blocking adhesion of <i>H. pylori</i> , preventing the sequelae of chronic gastric infections
[167]/2010	~2-5 μm	dex-HEMA-MAA (-), dex-HEMA-DMAEMA (+) microgels	Emulsification	SOPC/DOPA(-4°C) SOPC/DOTAP(+) DOPE(-16°C)/DOTAP(+)	Lipid film hydration/sonication	Water	Biodegradable, self-exploding under degradation	-
[168]/2011	~0.3 μm	Anchored PNIPAA/NIPAA-AA core-shell microgels/MB microgels	Precipitation polymerization	POPC (-2°C)	Lipid film hydration/extrusion	TRIS (pH7.2)	Temperature, pH sensitivity	Fluorescent probe
[20]/2012	1-15 μm	Sodium hyaluronate/PEGDA microgels	Emulsification	L-cethin (HSPC) (52°C)	W/o/w microemulsion	Water	Drying/wetting	Natural moisturizing factor (NMF), incubation?
[169]/2013	1-100 μm	PAAm/allylamine (+) or PAAm/AMPS (-) microgels/MB microgels	ISP	DOPC(22°C) or DOPG(-18°C)(-) or DOTAP (+)	Lipid film hydration/extrusion	HEPES (pH7.6)	Electrostatic liposomes/MG interactions	-

Abbreviations used in Tables 4.2 and 4.3: AA – acrylic acid, AHA –acetohydroxamic acid, AMPS – 2-acrylamido-2-methyl-1-propanesulfonic acid, BAA - bis-acrylamido acetate, BSA – bovine serum albumin, DCP – dihexadecyl phosphate, DMAEMA – dimethylaminoethyl methacrylate, DMPC - 1,2-dimyristoyl-snglycero-3-phosphatidylcholine, DMPE - 1,2-Dimyristoyl-snglycero-3-phosphoethanolamine, DOPA – dioleoyl glycerol phosphate (negatively charged), DOPC –1-2 dioleoyl sn-glycero 3-phosphocholine, DOPE - dioleoyl glycerol phosphoethanolamine (neutral), DOPG – dioleoylphosphatidylglycerol, DOTAP – dioleoyl trimethylammoniumpropane (positively charged), DPPC –1,2-dipalmitoyl-snglycero-3-phosphatidylcholine, DSPE – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, E-BIS – *N,N'*-ethylene-bis(acrylamide), EPC – Egg chicken L- α -phosphotidylcholine, dex-HEMA dextran hydroxyethyl methacrylate, HSPC – Hydro Soy L- α -phosphotidylcholine, MAA – methacrylic acid, MBA – *N,N*-methylenebisacrylamide, MG – microgel, NMF - natural moisturizing factor: serine (18.2%), glycine (9.1%), arginine (3.2%), glutamic acid (2.3%), tyrosine (0.5%), alanine (6.6%), pyrrolidone carboxylic acid (12%), urea (7%), sodium lactate (5%), and deionized water (to 100%), NPMA – 4-nitrophenyl methacrylate, PA – phosphatidic acid, PAA - polyacrylic acid, PAAm – polyacrylamide, PC – phosphocholine, PE – phosphatidylethanolamine, PDMAA – polydimethylacrylamide, PEDOT – poly(ethylene dioxythiophene), PEGDA – poly(ethylene glycol) diglycidyl ether, PI – phosphatidylinositol, PLA – Polylactide, PNIPA – poly(*N*-isopropylacrylamide), POPC – 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, PPG – dipalmitoylphosphatidylglycerol, PS – phosphatidylserine, PSA – sorbitol acrylate, PSS – poly(styrene sulfonate), TEGDM – tetraethylene glycol dimethacrylate, SOPC – stearyl oleoyl phosphatidylcholine (neutral), VI – 1-vinylimidazole,.

The research on engineering of lipobead has been focused on (i) enhancement of mechanical properties of the supported lipid bilayer by hydrophobic modification of the hydrogel core surface [8-11,17,84,150,161-165,168] and variation of the bilayer surface charge [6,7,167,169], (ii) encasing of biodegradable [22,143,149,151,154,167], temperature and pH [6,16,17,19,84,168,158,160, 152,157,159] sensitive hydrogel cores, and (iii) entrapment of biologically active agents and drugs such as gold nanoparticles [143], hemoglobin [152,153], antigens [5,19,157], ATP [11], transmembrane receptors [165], hydrophilic solutes and fluorescent probes [8,10,17,168], doxorubicin [6,7], moisturizing factor [20], interleukin-2 [145] into lipobeads. The *in vivo* animal studies have been performed to investigate the potential utility of the lipobeads for combination drug delivery administered intramuscularly [157] and intravenously [22].

Polymerization within liposomal nano-/microreactors

Effectors of the lipid bilayer stability and permeability

It is the stability and permeability that are the two main properties of a lipid bilayer, which should be governed in the course of lipobeads preparation. Mostly, this is the matter of the lipid bilayer fluidity, which depends on temperature and membrane composition. Bilayers undergo a change from the liquid to the gel (solid) state at the so-called lipid (or order-disorder) phase transition temperature (T_t) characteristic to a phospholipid used (Figure 4.7). In the liquid-crystalline “disordered” state, the membrane is fluidic, namely: (i) both alkane chains and head groups of phospholipids are more flexible than in the solid “ordered” state, (ii) the area lipids occupy becomes greater by changing from a 0.48 nm²/head group to 0.7 nm²/head group, i.e. bilayer expands, (iii) lateral diffusion of phospholipids in the plane of the bilayer and rotation of lipid molecules around C-C bonds accelerate, (iv) transverse “flip-flop” migration of lipids from one monolayer to the other side of the bilayer becomes more probable.

As a result, one can expect that above T_t a lipid bilayer is more elastic (favorable for formation of unilamellar membrane) and less sealed (unfavorable for gelation within liposomal interior) than below T_t . Figure 4.7 outlines the factors affecting the lipid phase transition temperature, which are crucial for lipobead engineering. It is known from the properties of naturally occurred membranes, that their stability and permeability can be varied by balancing composition of cholesterol and alcohols. Particularly, presence of cholesterol, strongly interacting with phospholipids, inhibits the

passive permeability of lipid membrane to water and small electrolytes and non-electrolytes. The extent of “sealing” directly depends on the amount of cholesterol present, up to moderate levels of cholesterol. However, at very high levels of cholesterol, pure cholesterol phase separates out and leads to an increased leakage through interfacial lipids and unstable aggregates of cholesterol. On the contrary, the insertion of anesthetic molecules, such as alcohols, into the membrane increases the membrane fluidity at a given temperature by depression of the lipid order-disorder transition temperature. Additionally, sphingolipids are commonly believed to protect the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane.

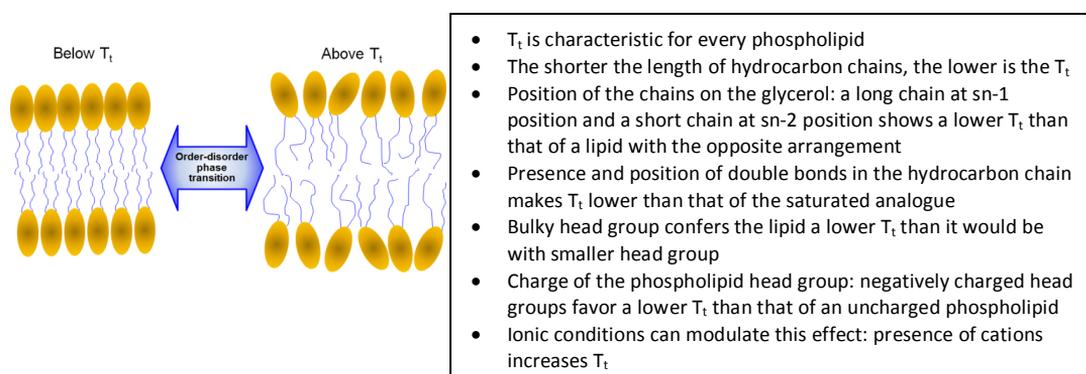


FIGURE 4.7

Structure of lipid bilayer above and below transition temperature and the factors affecting the lipid phase transition temperature

Table 4.2 shows that phospholipids with the T_t lower than room temperature are commonly used for polymerization within a liposomal reactor.

Common mechanisms of gelation

One can notice that gelation within a liposomal reactor includes both physical and chemical cross-linking reactions.

Thermal cross-linking. Some hydrogel cores are made of polysaccharides when temperature changes [143,146,151]. Indeed, agarose [146] and κ -carrageenan [151] are the temperature sensitive polysaccharides which structure in aqueous solutions undergoes a transition from a random-coil conformation to the cross-linked double helices upon cooling. Agarose is not biodegradable, but its combination with gelatin brings biodegradability [170].

Gelatin is a thermoresponsive protein, forming a reversible cross-linked network by cooling a water-based solution of the polymer below 35°C. The hydrogel can be liquefied by heating it to physiological temperatures.

Interestingly, κ -carrageenan, an anionic polysaccharide carrying one sulfate group, can be cross-linked both thermally (upon cooling) and ionotropically in the presence of divalent or monovalent cations [170]. Similar to alginate the degradation of carrageenan hydrogels is driven by the exchange of ions with the surrounding fluids.

Ionotropic cross-linking. In the course of ionic cross-linking within liposomal interior, the sections of the polymer backbone carrying the charge bind with ions of opposite charge. For example, when multivalent cations (e.g. Ca^{2+}) are added to a water-based alginate [146] or poly(ethylene

dioxythiophene)/ poly(styrene sulfonate) [155] solutions, they bind adjacent polymer chains forming ionic interchain bridges that cause a cross-linking. The pH driven cross-linking inside liposome is carried out by lowering the pH of aqueous solution of poly(acrylic acid) carrying carboxyl groups [19,157].

Chemical cross-linking. The greatest portion of works on gelation within liposomal reactor use photopolymerization to generate a strong covalently cross-linked hydrogel [16,22,84,142,152, 154,159-161]. The mechanism mainly relies on producing free radicals when irradiated by UV and simultaneous formation and cross-linking of the polymer chains. The degree of cross-linking depends on the presence and concentration of a cross-linker.

General scheme of gelation within liposomal interior

In general, preparation of lipobeads using vesicle interior as microreactors, includes a number of crucial steps, as shown in Figure 4.8. First of all, encapsulation of hydrogel-forming components into liposomes can result from hydration of lipid cast film formed upon solvent evaporation [22,84,142,143,146,150-152,154], electroformation [16,149,159,160], or rapid phase evaporation [19,157]. The size of liposomes ensures the final size of lipobeads. If one aims at lipobeads of a 100-nm diameter, the liposomal formulations should be sonicated or extruded through a nanopore filter of a needed pore size. Another approach to the formation of pre-lipobeads of a controlled size is based on the hydrodynamic focusing of the stream of liposome precursor solution by the flow of hydrogel forming solution within a microfluidic device [158]. Although the microfluidic-directed approach and electroformation are very elegant methods, their productivity should be assessed with regards to pharmaceutical applications.

Secondly, it is important to prevent cross-linking or polymerization outside liposomes. This can be done by several methods, such as a 5- to 20-fold dilution [16,22,84,149,152,154,159,160], gel filtration [142,158], centrifugation and dialysis [19,150,146,157], or introduction of polymerization scavengers (e.g., ascorbic acid [156]) into the extravesicular space. In addition, hydrogel forming solution as well as cross-linker and initiator can be microinjected directly into the internal compartment of a giant unilamellar phospholipid vesicle (GUV) [155].

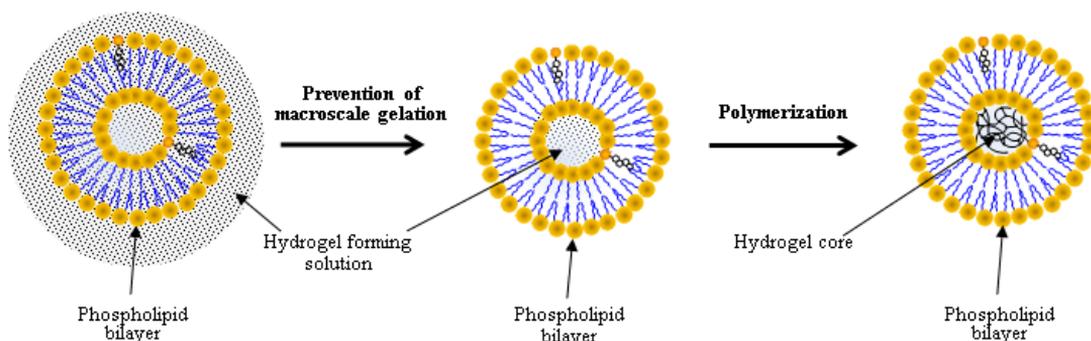


FIGURE 4.8

A “futuristic” view on the synthesis of hydrogel core within vesicle interior

The third step is gelation of the hydrogel forming solution entrapped within the closed lipid bilayer

that should be initiated in accord with one of the mechanisms outlined in the Section “Common mechanisms of gelation” of this chapter.

Finally, the formulation has to be washed from unreacted chemicals using centrifugation and/or dialysis. In the course of this step, the required medium external to the lipobeads can be introduced. If necessary, the prepared lipobeads can be dried by gentle evaporation in temperature gradient to be stored at 4°C.

Commonly, a hydrophilic drug cargo is added as a component of the hydrogel forming solution and incorporated into the intravesicular space before gelation started. As Table 4.2 shows, so far only chemically cross-linked hydrogel cores were tested with regard to drug loading.

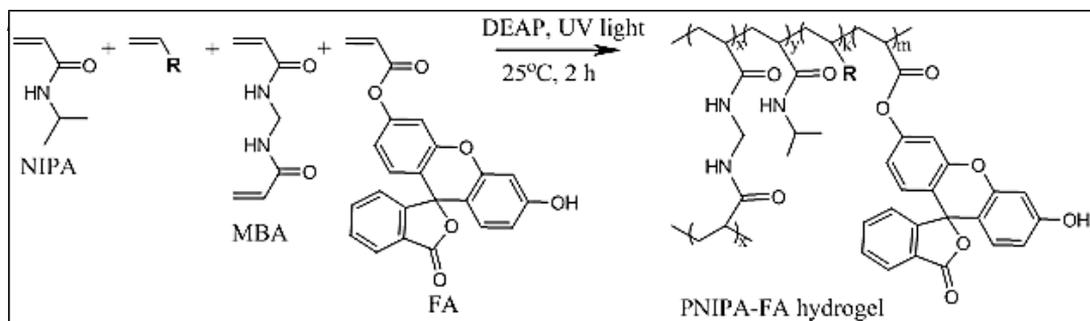


FIGURE 4.9

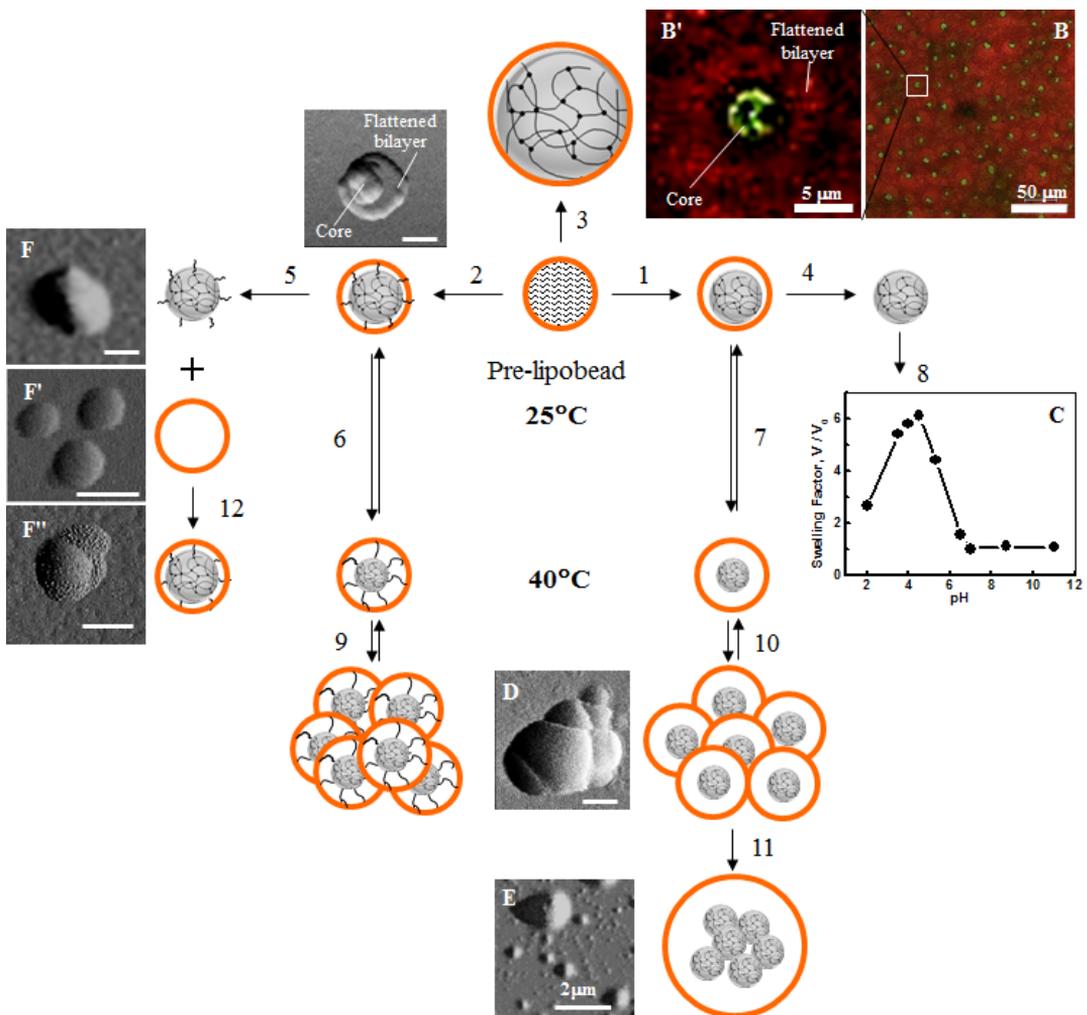
Reaction scheme and structure of PNIPA hydrogel core

The reaction scheme within a liposomal reactor includes polymerization and cross-linking of the main monomer as well as co-polymerization of different moieties providing stimuli responsiveness, hydrophobic modification, fluorescent staining for imaging, etc. (Figure 4.9).

Poly(*N*-isopropylacrylamide)(PNIPA) is a classic example of temperature sensitive polymer with the Lower Critical Solution Temperature (LCST) in the range of physiological temperatures. Cross-linked by *N,N*-methylene-bis-acrylamide(MBA), PNIPA forms a temperature sensitive three-dimensional polymer network. A residue R can bring either ionic (pH) sensitivity to the hydrogel, e.g., cationic 1-vinylimidazole (VI) or anionic acrylic acid (AA), or hydrophobic modification, e.g., *N*-(n-octadecyl)acrylamide. Fluorescein o-acrylate (FA) is a good candidate as a fluorescent label for hydrogel. This reaction has been proven to work reliably on macro- (bulk gel), micro- (giant lipobeads), and nanoscales (nanolipobeads).

Figure 4.10 shows the properties of lipobeads revealed at different steps of their preparation.

When a liposome containing a hydrogel forming solution with a photoinitiator (diethoxyacetophenone, DEAP) is exposed to UV light, free radical polymerization initiates and proceeds yielding the so-called non-anchored (process 1) or anchored (process 2) lipobeads. On the nanometer scale, bipartite structure of lipobeads is confirmed by AFM, which provides a sufficient resolution to distinguish between the hydrogel core and lipid bilayer (image A). On the micrometer scale, the structure of giant lipobeads (GLBs) can be directly observed under optical, fluorescence or confocal microscopes. Dual-color fluorescence images of GLBs fabricated by UV polymerization of PNIPA within giant vesicles containing a fluorescent phospholipid (process 3) are shown in Figure 4.10 (images B and B').

**FIGURE 4.10**

Gelation within liposomes and properties of PNIPA-VI lipobeards: see text for details.

All unlabeled scale bars are 100 nm

The green signal indicates the presence of hydrogel cores (FA was covalently attached to the polymer chains), whereas the red signal corresponds to ammonium salt of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod B-PE) present in the lipid bilayers. It is evident that all gel-cores are coated with lipid layers, which flattens on the glass slide surface. Polymerization within liposomes is additionally proved by visualization of nanogels (image F) obtained by the removal of the bilayer using a detergent (process 4 for non-anchored nanogels, process 5 for anchored ones). As revealed by Dynamic Light Scattering technique (DLS), PNIPA-VI nanogels are temperature and pH sensitive: their volume decreases ~ 8 -fold when temperature changes from 25°C to 40°C (processes 6 and 7) and increases ~ 6 -fold as pH changes from 7 to 4.5 at room temperature (process 8, image C). Moreover, DLS analysis shows that lipobeards prefer to aggregate in the course of thermal collapsing of the hydrogel core (processes 9 and 10). Interestingly, the anchored lipobeards do not fuse upon aggregation (image D) and

reversibly disaggregate when nanogels swell back at room temperature (process 9). In contrast to the anchored lipobeads, aggregation of the non-modified lipobeads at temperatures higher than the volume phase transition temperature $T_v \sim 32^\circ\text{C}$ (processes 10) appear to be irreversible due to their fusion (process 11) into the “giant” lipobeads shown in the image E.

Importantly, the nanogels once extracted from lipobeads exhibit a strong compatibility with phospholipid bilayer: the phospholipid bilayer spontaneously self-assembles around a nanogel (image F) upon mixing (process 12) with liposomes (image F') to form secondary lipobeads (image F''). This finding justifies the other method for lipobeads preparation: nanogel/liposome mixing.

Hydrogel/liposome mixing

Compatibility of nanogels and phospholipid bilayer is a key property in the context of lipobeads preparation by hydrogel/liposome mixing. It has been demonstrated by DLS and AFM [172] that hydrophobic modification of the nanogels is not required for spontaneous formation of the bilayer on their surface. Together with the other studies [163] these findings presume that hydrogel/lipid bilayer is an energetically favorable structure.

In accord with the general scheme of hydrogel/liposome mixing (Figure 4.11), the hydrogel particles and liposomes should be prepared first. In contrast to gelation within a liposomal reactor, the final size of lipobeads will be defined by the size of hydrogel particles prepared before mixing with liposomes.

As follows from Table 4.3, there are only a few papers that deal with nanogels to prepare lipobeads on the nanometer scale: one group employed a high pressure homogenizer to crash bulk polysaccharide hydrogel down to nanosized particles [161-164], the other group used nanogels extracted from liposomal reactors [12-15]. In principle, emulsion polymerization enables preparation of hydrogel particles with a diameter less than 150 nm. However, there is a problem of complete removal of the residual materials. In the absence of an added surfactant, the method is called precipitation polymerization. With the latter two methods, the lipobeads of 1- μm diameter are produced [6,17,20,143,167,168]. To prepare giant lipobeads with a diameter up to hundreds micrometers, the inverse suspension polymerization (ISP) method is commonly applied [8-11,165,166,169].

Generally, the method of phospholipid vesicles preparation is not critical. However, in the most cases (Table 4.3), conventional liposomes prepared by the lipid film hydration followed by sonication or extrusion are used for lipobeads' fabrication.

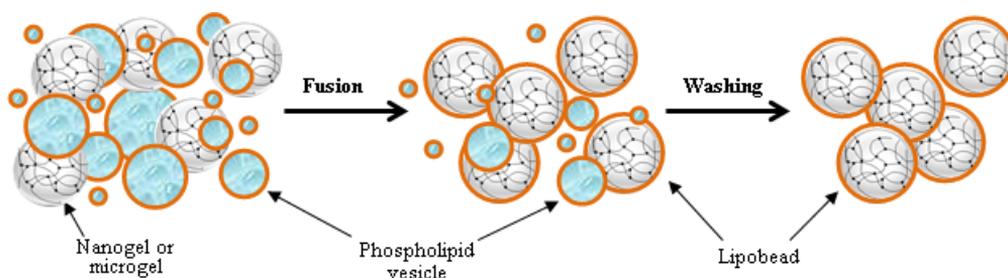


FIGURE 4.11

Schematic of the method for preparation of lipobeads using hydrogel/liposome mixing

Whatever the method of liposome production is, there are many ways to bring them into contact with hydrogel particles including (i) mixing hydrogel particles and liposomes, (ii) addition of hydrogel particles into dried lipid film before hydration, and (iii) hydration of the lipid film by aqueous suspension of hydrogel particles. To enhance the lipid bilayer formation around hydrogel particles, the procedures such as hand shaking, vortexing, pipette agitation, centrifugation, freezing-thawing, heating-cooling or their combination can be used. The fusion of liposomes will be more advanced at temperatures higher than the gel-liquid phase transition temperature of the phospholipid used. Moreover, depending on the electrostatic interaction between bilayer and hydrogel, the liposomes can adsorb on the particles surface, diffuse inside, or/and fuse on the surface with formation of lipobeads [169].

Usually, free liposomes are washed out by centrifugation or removed by ultrafiltration or dialysis. Finally, the lipobeads can be dispersed in a buffer with pH ranged from 7 to 8 or distilled water.

Giant lipobeads

On the one hand, the most effective size of the nanoparticle-based drug delivery systems is around 100 nm [171], because of its relevancy for intravenous administration: the particles of this size are not trapped in the blood capillaries ($\sim\varnothing 5-8\ \mu\text{m}$) and stay longer in the systemic circulation due to a lower risk for reticuloendothelial system uptake and their ability to escape processing in the liver and kidney for several circulation cycles. On the other hand, lipobeads of the size greater than diffraction limit of light microscopy ($\sim 1\ \mu\text{m}$) are attractive models for drug delivery systems, because they are easy to observe directly using optical, fluorescence or confocal microscopies for both controlling their stability and permeability in the course of preparation and modeling the mechanisms of drug loading and controlled release. Giant lipobeads can be prepared by either polymerizing the interior of giant vesicle filled with a hydrogel-forming solution or mixing microgels with giant vesicles liposomes.

Polymerization inside liposomal reactor

Giant vesicles filled with hydrogel forming solution containing monomers (NIPA and FA), a cross-linker (MBA), and a photoinitiator (DEAP) are formed by gentle hydration method. Lipid bilayer consists of a phospholipid and cholesterol in molar ratio of 9:1. A phospholipid with a higher order-disorder phase transition temperature (Hydro Soy L- α -phosphatidylcholine, HSPC, $T_t = 52^\circ\text{C}$) is used in order to facilitate the formation of giant vesicles by increasing temperature, as the lipid bilayer above T_t is in a liquid state and to prevent leakage of the hydrogel forming solution from a microreactor, as the lipid bilayer is in a solid state (less permeable) at room temperature. The fluorescent probe Rhod B-PE is added to provide fluorescent staining of the lipid bilayer formed. The results on polymerization within the giant vesicles are presented in Figure 4.12.

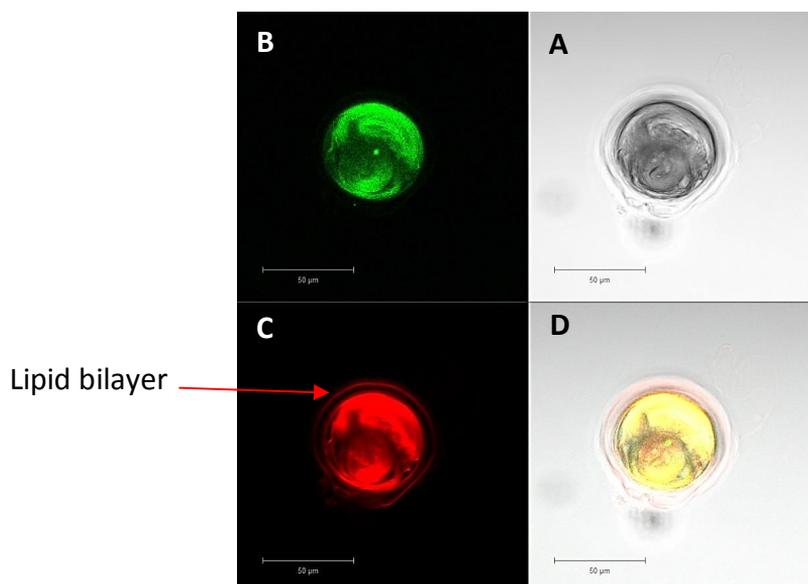
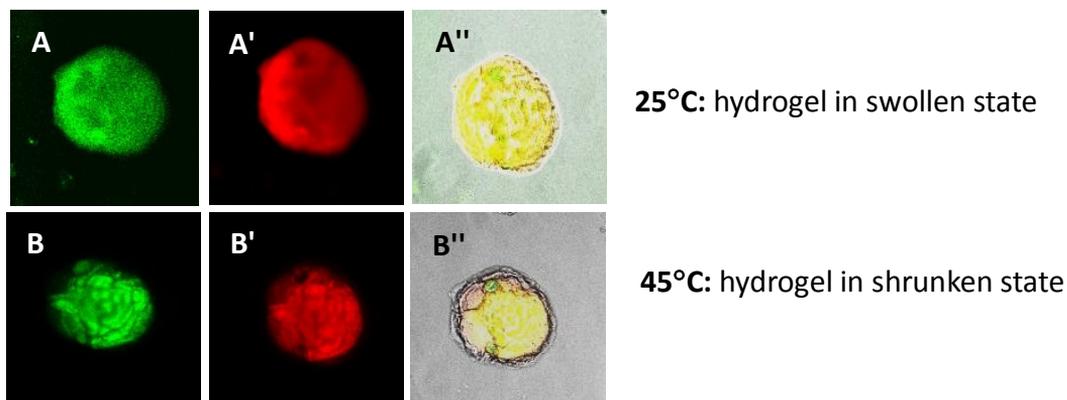


FIGURE 4.12

Giant liposomes filled with poly(*N*-isopropylacrylamide-co-fluorescein-o-acrylate) hydrogel (PNIPA-co-FA): A – bright field image, B – hydrogel core, the green image originates from Fluorescein-o-acrylate (FA, 488 nm excitation) covalently attached to PNIPA network, C – flattened phospholipid bilayer, red image originates from Rhod B-PE (555 nm excitation) covalently attached to the heads of phospholipid, D – overlap of all three images

Giant lipobeads (GLBs) in the range of 5 to 100 μm are observed (Figure 4.12) in a good contrast under both bright field (A) and fluorescence (B, C) modes of the laser scanning confocal microscope (LSM 700, Zeiss). Green colour of the central part (B) demonstrates the presence of hydrogel core, since it originates from the FA dye that was co-polymerized with PNIPA hydrogel. The red tiny external layer can be distinguished around GLB in Figure 4.12C. Red image originates from the fluorescent phospholipid, Rhod B-PE, added in the course of giant vesicles formation. The presence of red colour in the central part (C) can be explained by flattening of GLBs on the surface of glass slide to cause a collapse of external layer inside and/or multilamellar origin of the lipobeads. Despite the revealed multilamellarity, the GLBs exhibit a substantial stability: they have withstood multiple centrifugations and numerous dilutions by distilled water.

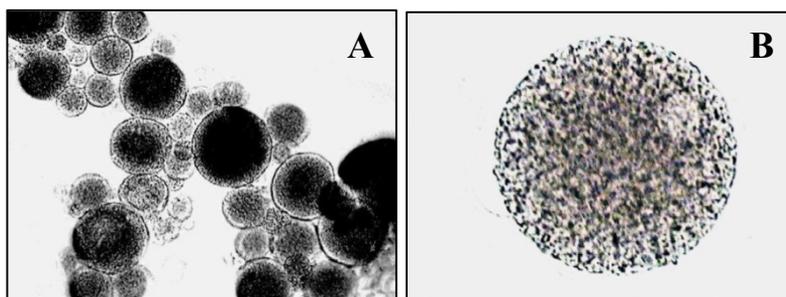
PNIPA hydrogel is known to exhibit a reversible volume contraction upon temperature increase above the volume phase transition temperature ($\sim 32\text{--}37^\circ\text{C}$ in water). Figure 4.13 shows the same particle at 25°C (A, A', A'') and 45°C (B, B', B''). The shrinking of the central part followed by a decrease in the total size of GLB. These observations are in contradiction with the data on nanolipobeads [84,148,152] showing no variation in the lipobead and liposome sizes with the change in temperature from room temperature to 40°C , whereas PNIPA nanogels extracted from the lipobeads shrink considerably. Possible explanation may be in that the nanometer-sized lipid bilayers with a greater curvature are stiffer and do not couple to the gel surface.

**FIGURE 4.13**

The same giant lipobead below (A) and above (B) the volume phase transition temperature of PNIPA-co-FA hydrogel ($T_v \sim 37^\circ\text{C}$): A, B – green images originated from FA covalently attached to the PNIPA network within the core; A', B' - red images originated from rhodamine B covalently attached to the heads of PE; A'', B'' - overlap of bright field and fluorescence images

Microgel/liposome mixing

The ultimate goal to this end is to decrease the number of steps in preparation of lipobeads. Giant lipobeads can be used to examine whether a spherical microgel can be covered by a lipid bilayer even if it is mixed with multilamellar (not unilamellar) vesicles. Moreover, a recently proposed method for preparation of liposomes [173], comprising the injection of a phospholipid solution in ethanol into hot water, can be compared with the conventional one, based on lipid film hydration. The PNIPA-FA microgels with fluorescence ability are synthesized according to the reaction scheme presented in Figure 4.9 using an inverse suspension polymerization method. Optical micrographs indicate (Figure 4.14) that the size of PNIPA-FA microgels ranges from 2 to 150 μm .

**FIGURE 4.14**

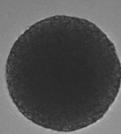
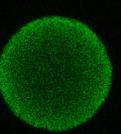
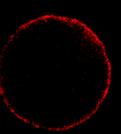
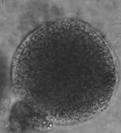
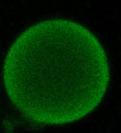
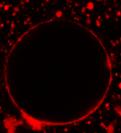
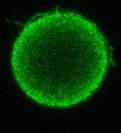
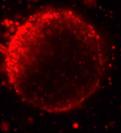
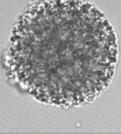
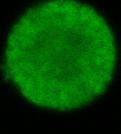
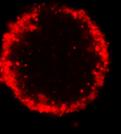
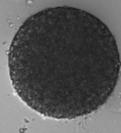
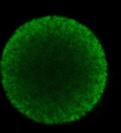
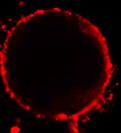
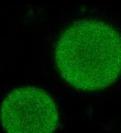
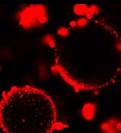
The bright field images of PNIPA-co-FA hydrogel spheres after washing: A – 200x, B – 1000x

Giant multilamellar vesicles (GMV) are prepared by two methods: (i) the conventional method of lipid film hydration and (ii) a recently proposed method [173], comprising the injection of a phospholipid solution in ethanol into hot water. The lipidic formulations contain either HSPC ($T_t = 52^\circ\text{C}$) or Egg chicken L- α -phosphatidylcholine (EPC, $T_t = -10^\circ\text{C}$). If cholesterol is added, its amount is

adjusted to the phospholipid/cholesterol molar ratio of 9:1. Moreover, to visualize the lipid coat, fluorescent phospholipid RhodB-PE is added. Totally, six lipidic formulations are mixed with the microgels suspensions and incubated overnight with three freezing-thawing (for EPC) or heating-cooling (for HSPC) cycles. After washing by centrifugation, six types of lipobeads are systemized in Table 4.4 and visualized using confocal laser scanning microscopy.

TABLE 4.4

Lipidic formulations mixed with microgels and confocal images of the resultant giant lipobeads

METHOD OF GMV PREPARATION	SAMPLE #	LIPID COMPOSITION		T_v , °C	MICROSCOPY MODE		
		PL	CHOL		Bright field	Hydrogel core	Lipid bilayer
LIPID FILM HYDRATION	1	EPC	-	-10			
	2	EPC	+	-10			
INJECTION OF ETHANOL SOLUTION OF PHOSPHOLIPID INTO HOT WATER	3	EPC	-	-10			
	4	EPC	+	-10			
	5	HSPC	-	52			
	6	HSPC	+	52			

The first observation is that microgels have a lipid coat in all cases. Nevertheless, homogeneity of the lipid layers depends on the extent of its fluidity. Indeed, the formulation #1, which corresponds to the GMVs prepared by hydration of the lipid film consisting of the phospholipid with the lowest T_t and without cholesterol, exhibits a continuous (probably unilamellar) lipid bilayer. A worse

situation is in the case #6, in which the GMVs are made of the most solid lipid bilayers containing cholesterol and the phospholipid with T_c much higher than room temperature. Besides lipid coating, one can notice many unfused vesicles adsorb onto the surface of microgels. Therefore, one can conclude that multilamellarity and stiffness of GMVs are the factors that make it virtually difficult to separate the lipobeads from unfused and unbound vesicles, because of their similar size and density.

The most heterogeneous formulation #6 was sonicated for 2 hours and incubated with microgels overnight. The confocal microscopy images of two lipobead fabrications made of PNIPA-co-FA microgels mixed with GMVs (#6) either before (A) or after (B) sonication are presented in Figure 4.15. Their comparison explicitly shows that the smaller unfused vesicles can be readily washed out from the lipobead suspension by a low-speed centrifugation, so that a microgel sphere is completely covered by a homogeneous lipid layer.

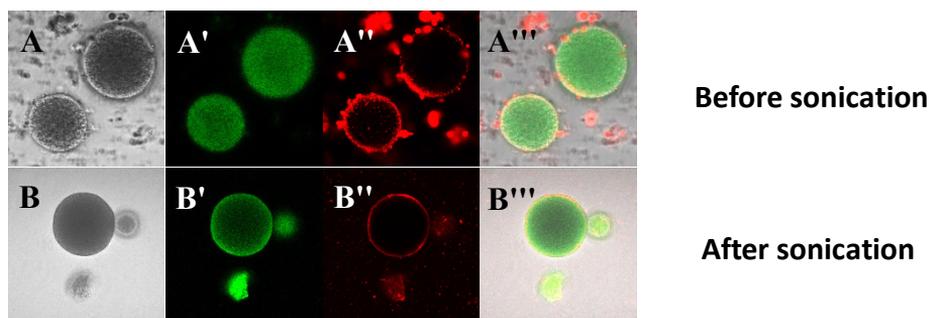


FIGURE 4.15

The bright field (A, B) and confocal scanning (A', A'', B', B'') microscopy and their overlap (A''', B''') images of PNIPA-co-FA hydrogel microspheres mixed with the giant multilamellar vesicles (formulation #6 in Table 4.4) before (A) and after (B) sonication

The experiments on giant lipobeads show that injection of ethanol solution of phospholipid into hot water is a promising method for preparation of lipidic formulations in comparison to the conventional lipid film hydration. In fact, exclusion of the time-consuming steps of lipid film formation and hydration may allow one to reduce the time for the scaled fabrication of lipobeads from days to hours.

Future Applications of Lipobead-Encapsulated Drugs

Loaded lipobeads – Encapsulation

It is interesting that one of the key reasons for the chemotherapeutic success of liposomal doxorubicin is highly efficient encapsulation and good retention, the properties resulted from its ability to precipitate inside liposomes [109,112,174]. The estimated doxorubicin concentration in liposomes is over 100 mg/mL (0.2 M) [174]. Does a lipobeadal drug delivery system have an advantage in loading capacity against liposomal drugs?

The data systemized in Table 4.2 indicate that in all cases of loaded lipobeads prepared by polymerization within a liposomal reactor, a drug was introduced into the aqueous phase followed

by rehydration of a lipid film and further polymerization within a liposomal reactor. The major challenge of this scheme of drug loading might be the damage to the drug by toxic ingredients of the hydrogel forming solution (if any) and/or high temperature and UV radiation initiating polymerization. This approach may be especially problematic for encapsulation of a protein drug, because of denaturation. Nonetheless, it has been reported that antigen model (BSA) [19] or combination drug [157] of protein antigen (Pfs25) and oligonucleotide sequence (CpGODN)¹ encapsulated into pH-cross-linked PAA hydrogel core of lipobeads remain intact and active. Moreover, encapsulation efficiencies of lipobeads are shown to be by 10% higher than those for liposomal carriers. A high encapsulation efficiency of lipobeads has been demonstrated also for hemoglobin [152,153], which withstands the conditions of free radical polymerization and UV radiation. The other example of successful co-encapsulation of protein and small molecule drugs into lipobeads under UV polymerization has been examined in [22].

In the case of lipobeads prepared by hydrogel/liposome mixing (see Table 4.3), the only drug loaded into microgels was doxorubicin [8,9]. Encapsulation was performed before lipobeads formation by soaking the dry hydrogel particles in a drug-dissolved solution. The drug diffuses inside in the course of the polymer network swelling and mesh size increase. Further mixing of hydrogel particles with liposomes encapsulates the drug into lipobeads. As a result, the unbelievably high doxorubicin concentration of ~2 M, which is 10-fold the concentration in liposomes [174], was achieved.

Encapsulation of a protein drug into hydrogel particles before lipobeads formation can be performed either by formation of a hydrogel particle in the presence of a protein drug or by incubation of the pre-formed hydrogel particles in a protein solution. The first approach again could be problematic due to a danger of protein denaturation. The second approach is limited by the size-exclusion effect resulting in a lower loading concentration of proteins. However, encapsulation of proteins into microgels is a promising tool to increase the amount of drug loaded in a pre-lipobead (loading capacity) by using the “intelligent” properties of polymer networks (swelling/shrinking ability in response to stimuli) [175].

New mechanisms of controlled drug release

Drug release from lipobeads, conventional liposomes, and hydrogel particles

A drug release profile (the amount of drug released into the bloodstream over time) depends on the properties of the drug itself and drug carrier system. Even a few available examples of drug-encapsulated lipobeads show that the additional element in their structure, the hydrogel core, can significantly prolong the release time for both high molecular weight (e.g., proteins) and small molecule (e.g., doxorubicin) drugs as compared to conventional liposomes and uncoated hydrogel particles. Indeed, the characteristic time for release of 50% (D_{50}) of BSA (Mw 66 kDa) from 1- μ m lipobeads (~ 11 days) is 10-fold of that from 1- μ m liposomes (~1 day) [157], whereas for a lighter protein interleukin-2 (Mw 17 kDa) [22] D_{50} equals 8 hours, 16 hours, and 52 hours for nanogels (\varnothing 150 nm), liposomes (\varnothing 100 nm), and lipobeads (\varnothing 120nm), respectively, indicating the slower release of the protein drugs from lipobeads. In comparison, the characteristic time(D_{50}) for release of doxorubicin from uncoated microgels (~ \varnothing 6 μ m) was estimated [8,9] to be about 1.5 min, whereas the release of doxorubicin from lipobeads was not detected at all within this time scale.

¹ CpG oligodeoxynucleotide (CpGODN) had the sequences GCTAGACGTTAGCGT and TCAACGTGA.

Different applications require different release profiles, and bi-compartmental structure of lipobeads brings more options to change the concentration profile of a released drug from a steep rise (burst release) and a cyclic variations (pulsatile release) to a gradual increase up to the value within the therapeutic window is reached (sustained or controlled release). Of particular importance is the capability of lipobeads to provide a better-sustained release which is the most desirable but more difficult mode to achieve and maintain.

The specific properties of lipid bilayer have already been discussed (discussed in the section "Effectors of the lipid bilayer stability and permeability"). Let us consider the novelty the hydrogel core can bring with regard to drug release mechanisms. Undoubtedly, an advanced property of polymer networks is their responsiveness to environmental stimuli. Depending on possible responses of the hydrogel core (swelling, contraction, and degradation), three mechanisms of drug release from lipobeads could be developed in the future.

"Sponge-like" mechanism

Figure 4.16 illustrates the "sponge-like" mechanism of drug release from lipobeads. Therein, hydrogel core initially is in a swollen state. Therein, encapsulated drug molecules release for a prolonged period of time as compared to conventional liposomes. When the environment changes (temperature, pH, etc.), the polymer network shrinks, so that the hydrogel core like a squeezed sponge releases the loaded drug into the space between gel and lipid membrane, and the drug diffuses through the membrane outside the lipobead. This mechanism provides the way of a gradual increase in the rate of drug release in response to for example temperature change.

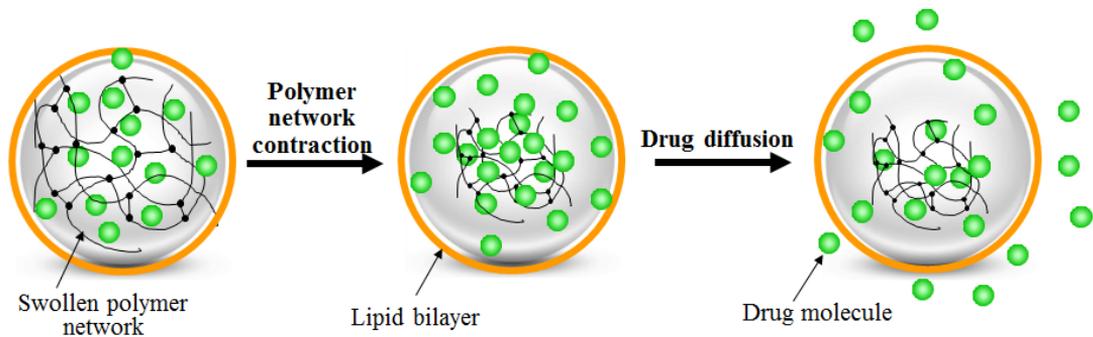


FIGURE 4.16

"Sponge-like" mechanism of drug release from lipobeads

"Poration" mechanism

In Figure 4.17, the hydrogel core initially is a shrunken state and drug molecules are trapped more tightly within the polymer network, so that their release can be even more suppressed in comparison with conventional liposomes. When the environment changes (temperature, pH, etc.), the polymer network swells so much that the volume of hydrogel core becomes greater than the space provided by the closed lipid bilayer. Therefore, a "growing" hydrogel core causes disruption of the lipid bilayer and pore formation ("poration") resulting in the drug release through the pores.

This mechanism provides the way of a drastic increase in the rate of drug release in response to stimuli.

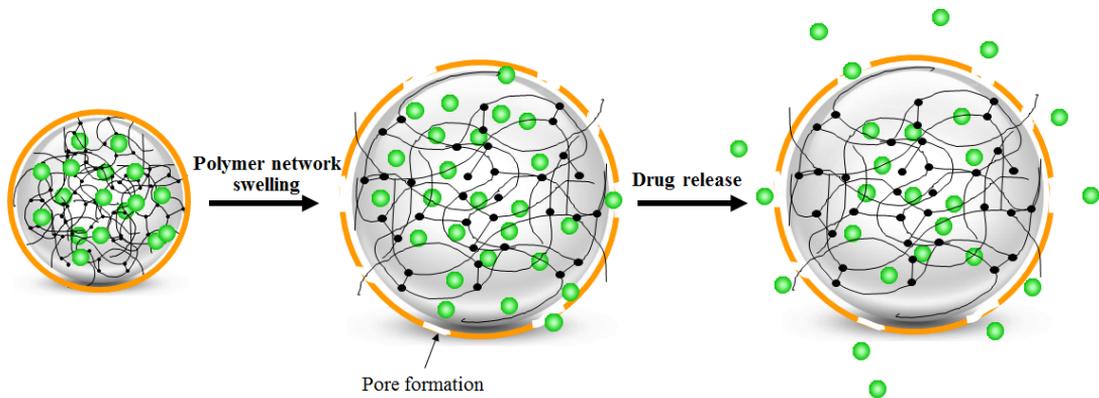


FIGURE 4.17
“Poration” mechanism of drug release from lipobeads

“Burst” mechanism

The “exploding” lipobeads have been discovered [167] as a byproduct of biodegradation of microgels covered with phospholipid membrane. As schematically outlined in Figure 4.18, if a polymer network degrades, for example, the interchain cross-links can be cleaved by hydrolysis, the swelling pressure inside increases, because the degradation products are unable to diffuse through the lipid membrane even it stretches. At some point, the internal pressure becomes sufficient to break the membrane. As a result, encapsulated drug falls out of lipobeads with the maximal release rate (burst release).

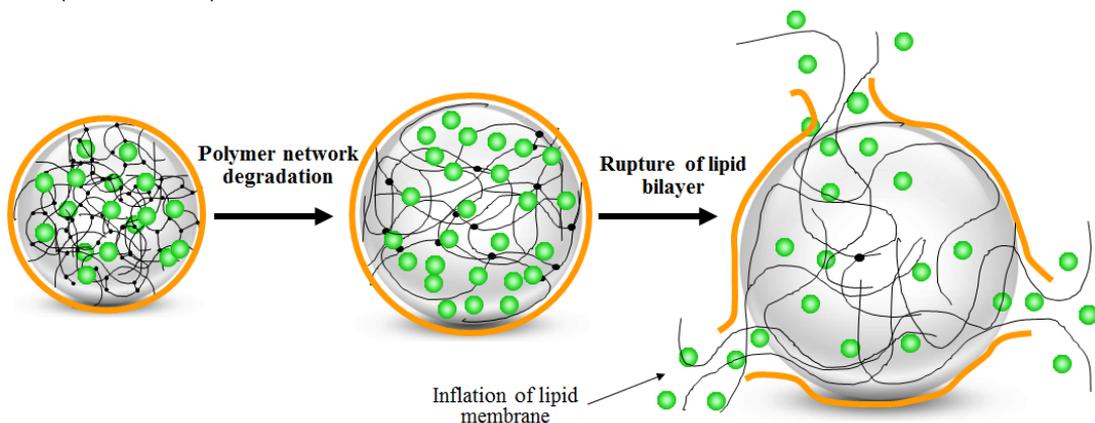


FIGURE 4.18
“Burst” mechanism of drug release from lipobeads

Two types of temperature-sensitive hydrogels

As temperature is an environmental property easy to vary, control, and predict in the practical

schemes of drug delivery, it is worthy to outline the two types of polymer networks behavior in response to temperature changes. Typically, the thermo-responsive hydrogels are classified as having either positive or negative volume phase transition with a characteristic temperature (T_V) [176]. Hydrogels exhibiting positive volume phase transition (“thermophilic” hydrogels) swell upon heating and should be used in the delivery systems with “poration” mechanism. In contrast, hydrogels exhibiting negative volume-phase transition (“thermophobic” hydrogels) collapse upon heating and are suitable for drug carriers using the “sponge-like” mechanism.

Of particular interest are the thermo-sensitive hydrogels of both types with the volume phase transition temperature T_V within the physiological range (37 – 50°C). The “thermophobic” hydrogels have been studied the most, and a popular example is PNIPA which T_V can vary close to the desirable range [177]. In contrast, “thermophilic” behavior in water is not very common for synthetic polymeric materials. The “thermophilic” gels are not well studied and they need complex preparation processes, and likely, because of that, lipobeads with a “thermophilic” hydrogel core have not been attempted yet. Nevertheless, very recently, a number of “thermophilic” hydrogels showing a positive thermosensitive volume change under physiologically relevant conditions have been fabricated [178-180].

Combination and multifunctional drug delivery systems

Drug combinations in lipobeads

Only two formulations tested as combination drugs delivered by lipobeads (Table 4.2) show the following advantages with respect to liposomal delivery systems.

In the first set, protein (Pfs25) and oligonucleotide (CpGODN) have been simultaneously encapsulated into lipobeads [157]. The recombinant protein Pfs25 expressed in *Pichia pastoris* is a leading antigen of blocking stage potential as a vaccine to block malaria transmission by mosquitoes. Antigen Pfs25 has a poor immunogenicity and needs an enhancer of immunological recognition. Unmethylated CpG oligodeoxynucleotide (CpGODN) is a strong stimulator of immune response in mammalian hosts and acts as adjuvant improving immunogenicity of co-administered protein antigen as well as reducing the amount of protein required. CpGODN stimulates the immune system through a specific receptor TLR9. The immune activity of CpG can be monitored by following the levels of nonspecific and specific immunoglobulins, a variety of cytokines, gamma interferon (IFN- γ) and increased lytic activity (see [157] for references).

The results showed that (i) on 90 days antigen storage at 4°C the detected leakage was 26% and 5% from conventional liposomes and lipobeads, respectively, (ii) no macroscopic sign of adverse reaction (redness, swelling and formation of granulomas) at the site of intramuscular injection were observed for both conventional liposomes and lipobeads, (iii) lipobeads encapsulated with combination of Pfs25 and CpGODN showed the maximal immune response based on serum anti-Pfs25 profile of immunized mice, (iv) significantly higher levels of interferon- γ and interleukin-2 were detected in the spleen if mice immunized with lipobeads carried the drug combination.

In the second scheme [22], hydrophilic protein(interleukin-2, 17 kDa) and hydrophobic small molecule drug (SB505124, 335 Da) have been co-encapsulated into the hydrogel core of 120-nm lipobeads cross-linked by a free radical photopolymerization. Interleukin-2 (IL-2) belongs to the family of cytokines, soluble proteins that supposedly stimulate natural killer cells (NK) and enhance lytic activity against melanomas and renal cancer. However, efficiency of the IL-2 as an immunotherapeutic agent may be significantly reduced by the ability of tumor cells to secrete a number of immunosuppressive factors, such as the transforming growth factor- β (TGF- β) that

decrease local immune responses. SB505124 (SB) is a TGF- β antagonist that inhibits TGF- β receptor. The results of co-encapsulation and simultaneous sustained delivery of the drugs with drastically different properties show the following advantages of using lipobeads: (i) bioactivities of both SB and IL-2 are unaffected by the incorporation procedure (UV exposure in the course of polymerization), (ii) no toxicity is observed on intravenously administrated mice, (iii) biodistribution analysis of rhodamine-loaded lipobeads in healthy mice receiving intravenous administration reveals that the lipobeads primarily accumulate in lungs, liver and kidney, the heart and spleen are also reached though, (iv) in B16 lung metastatic animals, the highest accumulation of lipobeads and drug is found to occur in the lungs and liver, (v) lipobead-delivered combination immunotherapy drastically increases survival, (vi) combination therapy stimulates both innate and adaptive immune systems, (vii) in comparison to other delivery systems including liposomal, a significantly greater reduction in both tumor growth rate and tumor mass is observed after one week therapy of the B16/B6 mouse models of metastatic melanoma administered intravenously.

Combined multifunctional containers

As per Figure 4.10, collapsing of nanogel core at elevated temperature causes aggregation of lipobeads. The aggregation can be reversible or irreversible depending on whether lipid bilayer fusion occurs or not. If nanogels are modified by hydrophobic anchors, the surrounding bilayers do not fuse (process 9), so that the aggregates disassemble reversibly when temperature returns to the initial one. In contrast, if nanogels are not anchored, the surrounding bilayers can eventually fuse to form a greater lipobead (process 11), which is incapable of disassembling reversibly when temperature decreases.

Reversible and irreversible aggregation of lipobeads could be a key step for designing two types of combined multifunctional containers. In the system made of anchored lipobeads, the initial formulation may consist of two different drugs entrapped in different lipobeads (Figure 4.19A). Under switching condition 1, both drugs can be simultaneously delivered as one aggregate to the targeted organs in the body. At switching condition 2 or 3, either one or the other drug can be released in the desired order.

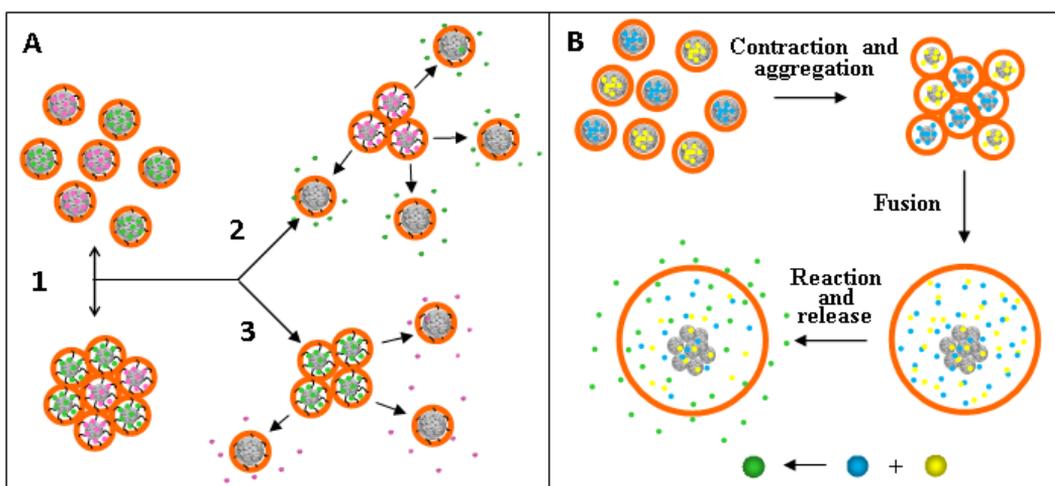


FIGURE 4.19

The combined drug delivery system based on reversible (A) and irreversible (B) aggregation of lipobeads

In the system based on irreversible aggregation of lipobeads (Figure 4.19B), several nanogels loaded with different pre-drug reagents are trapped under the same lipid membrane (“giant lipobeads”) to react inside without damaging the surrounding organs and to be delivered to the targeted site in one “giant” container able to release the final product controllably.

The fate of systemically administered lipobeads

Different administration routes including intravenous, intramuscular, pulmonary and topical could be suitable to deliver drugs by lipobeads, however, the peripheral intravenous injection would be the most reliable and reproducible route for their administration. The general scheme of lipobeads delivery at the target organ supposes to be similar to liposomal or polymeric delivery systems. After peripheral intravenous injection, lipobeads should withstand a number of environmental attacks (physiological and physicochemical) on the way to different organs via bloodstream.

Once entering the bloodstream, lipobeads run into a complex environment of the serum components (proteins, electrolytes, etc.) and immune system (macrophages, proteins of complement system, etc.). The results on preparation of lipobeads (Tables 4.2 and 4.3) clearly indicate that they can withstand physiological conditions and media. Interaction of lipobeads with plasma proteins could result in either leakage of their content or their removal from the blood circulation as exogenous pathogens. For example, it is reported for liposomes that the proteins of complement system [181] are able to produce lytic pores and enhance the release of liposomal content, whereas blood lipoproteins destabilize liposomes to increase the leakage of their payload [182]. The opsonins and dysopsonins are the other blood proteins which could be responsible for recognition of lipobeads and their enhanced uptake by the MPS (mononuclear phagocyte system) cells (neutrophils, monocytes, macrophages) [183-186]. Definitely, the physicochemical factors (size, charge, hydrophobicity, surface morphology, and composition) responsible for promoting lipobeads' leakage in and clearance from blood are the first target for study lipobeads as a drug delivery system in the future. Even just a few results available on the drug-encapsulated lipobeads (pegylated [22] or not [157]) have demonstrated their stability, biodistribution, toxicity, and therapeutic activity that are noticeably better than those for liposomes.

Further, the blood with lipobeads is pumped up by heart to different organs. Undoubtedly, the mechanical stability of lipobeads in the blood flow will be higher than that of liposomes, since in this construct a lipid bilayer is supported by hydrogel core and can be strengthened even more by anchoring. The capillaries with a diameter ranging from 2 to 10 μm constitute the first sieving constraint for the lipobead size. The particles of the size between 0.4 to 3 μm would mainly be captured by the liver macrophages. The lipobeads greater than 200 nm [187] would preferentially be filtered by the spleen. The smaller limit comes from the fact that particles less than 40 nm [186] should undergo clearance through metabolism in the liver and excretion through kidneys. Therefore, the diameter of lipobeads is supposed to be in a relatively narrow range from 50 to 180 nm for a longer retention in the bloodstream. Interestingly, it has been proven that formulations of lipobeads are the most reproducible in this range of sizes especially if prepared by polymerization within a liposomal reactor (see Table 4.2).

This range of sizes looks appropriate for lipobeads to exit systemic circulation. To reach interstitial space, lipobeads must cross a thin inner membrane of squamous endothelial membrane provided by the capillaries. In normal capillaries, the endothelial cells provide uninterrupted linings with typical gaps of 5 – 10 nm in size. In capillaries associated with pathologies such as tumor and inflammation, the gaps between endothelial cells are reported to vary from 100 to 780 nm for different types of cancer [188]. Due to rapid and imbalanced vessel formation, the tumor

neovasculature is chaotic, extremely heterogeneous and “leaky” [189]. The enhanced vascular permeability of the tumor capillaries is the first factor contributing to the phenomenon referred as the enhanced permeation and retention (EPR) effect [28,29]. The second factor of the EPR effect – an enhanced retention of lipobeads in the interstitial space – can be expected due to a poor lymphatic drainage in the tumor tissue, which results in a slower clearance of drug carriers and their accumulation in the interstitial space [190]. Biodistribution experiments have already performed in mice bearing a distant subcutaneous tumor and in mice with metastatic lung melanoma [22] to show accumulation of drug-loaded lipobeads both in the area surrounding the tumor as well as within the tumor itself [22]. Therein the payload is evident in the interstitial spaces between the tumor cells outside the vasculature.

In the interstitial space, lipobeads passively or actively target the cellular surface. Strategies of active cell targeting which has been proposed for liposomal carriers [127-130] could be applicable to lipobeads as well. A higher internalization of lipobeads by endocytosis could be achieved if their surfaces are decorated in accord with the methods for facilitation of intracellular delivery of the liposomal drugs reviewed in the section “Liposome-encapsulated drug carriers”.

The internalization of lipobeads into the cells can proceed via several mechanisms sketched in Figure 4.20. Omitting the details described in [191,192], phagocytosis provides the so-called “cell eating” mechanism by which larger lipobeads can be taken into and degraded within the cells. Using pinocytosis, the cells internalize the fluid surrounding the cell simultaneously with all substances (“cell drinking” mechanism), so that if lipobeads are in the fluid phase area of invagination, they would be taken up to form pinosomes inside. Different endocytic pathways can be distinguished in accord with the specific molecular regulators (not shown in Figure 4.20), such as the clathrin-mediated endocytosis, dynamin-dependent and dynamin-independent mechanisms, as well as receptor-mediated endocytosis. In addition, the mutual fusion of cell membrane and lipid bilayer of lipobeads [193] can occur at the cell surface with internalization of just the drug-loaded nanogels. Understanding the cellular entry of lipobeads, their intracellular trafficking, drug release and therapeutic action mechanisms are the future topics for studies on lipobeadal drug delivery systems.

The next levels of complexity

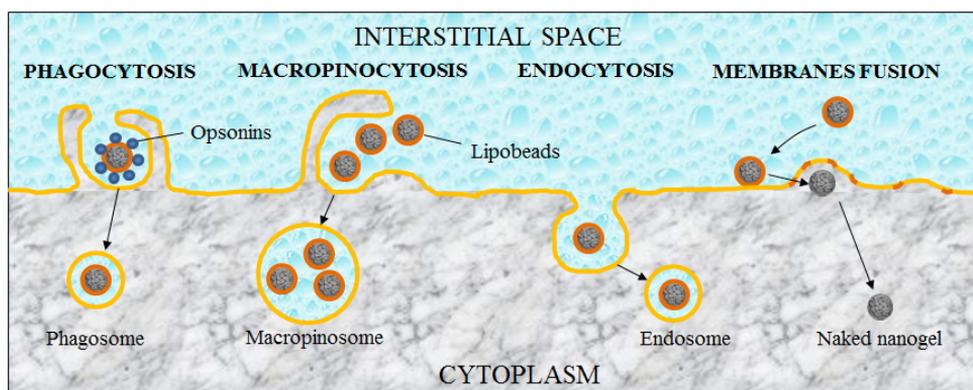


FIGURE 4.20

Possible mechanisms of lipobeads internalization into the cell

Proteo-lipobeads. The first proteo-lipobeads have been prepared when transmembrane receptors were reconstituted into the lipid bilayer of lipobeads and the receptors retained their native specific binding [165]. Recently, new proteo-lipobeads with a controlled orientation of the membrane protein and enhanced stability have been developed by modifying agarose beads with linkers, binding membrane proteins to the linkers, and surface coverage with phospholipids [194]. It has been shown that the lipobead incorporated cytochrome c oxidase is functional in terms of antibody binding and proton transport modulation. The increase in complexity of a lipobead structure is expected to bring about new benefits, such as tiny living cells mimicking mechanisms of drug release regulated by signaling.

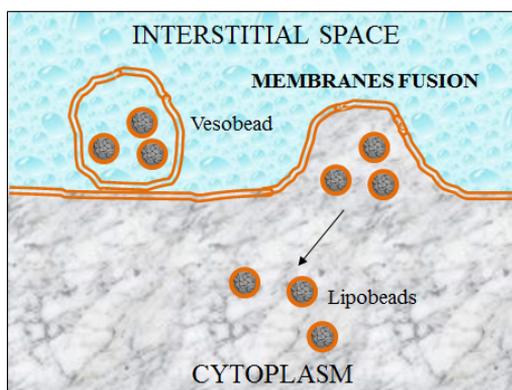


FIGURE 4.21

From vesobeads to cytoplasm: A likely mechanism of lipobeads internalization into the cell

Vesobeads. By analogy with liposomes encapsulating smaller liposomes known as *vesosomes* [195,196], liposomes encapsulating smaller lipobeads can be constructed and named as *vesobeads*. The structure of a vesobead resembles the structure of a macropinosome as depicted in Figure 4.20. Besides all advantages of conventional lipobeads discussed in this chapter, the multicompartmental structure of vesobeads will provide additional protection against degradation and leakage in bloodstream and greater biocompatibility. A new mechanism of internalization of lipobeads into the cells could be devised from the concept of vesobeads. As shown in Figure 4.21, if an external lipid bilayer of vesobead fuses with the cellular plasma membrane, a bunch of drug-loaded lipobeads are injected inside the cell.

Conclusions & Closing Remarks

The concept of lipobeads has been proposed about 30 years ago, however, lipobead-based drug delivery systems are still largely experimental. A possible reason for a limited number of studies on lipobeads published as yet could be in the lack of a comprehensive understanding of the advantages of these drug carriers versus the feasibility of their production. To all appearances, if the development of polymeric and liposomal drug delivery systems approaches its conceptual limit incapable of providing the desired level of functionality, the time has come to explore a qualitatively higher level of complexity, a combination of lipid bilayer and cross-linked polymer

network, which Nature uses to achieve workability, multifunctionality, and dynamism in living cells of different types.

Lipobeads demonstrate a combination of properties attractive for the next generation of drug delivery systems. First, they retain all the important benefits of liposomal drug carriers in one construct: efficient encapsulation of a wide variety of drugs, biocompatibility of the lipid bilayer, possibility of passive targeting to tumor or inflammation sites due to the controlled composition regulating the size and morphology, availability of the external surface as a modification site for the attachment of various ligands depending on the desired purposes (prolongation of circulation time, active targeting, etc.), no macroscopic signs of adverse reaction (redness, swelling and formation of granulomas) at the site of intramuscular injection.

The key properties that hydrogel core brings to the formulation are the tougher mechanical stability and environmental responsiveness. A polymer network containing up to 99.9% water behaves as a solid-like supporting cushion for the lipid bilayer. A polymer network responds mechanically (swelling/shrinking) and/or electrochemically (accumulating/releasing ions) to environmental changes. For example, a thermosensitive, cholesterol-free, and pegylated liposomes (ThermoDox®, Celsion Corporation, New Jersey, USA) in combination with hyperthermia have a clear advantage of fast drug release at the tumor site (breast cancer) [197], however, they still leak a considerable fraction of doxorubicin into plasma, which may explain their clinical toxicity. Definitely, lipobeads could provide similar thermosensitivity without leakage of an encapsulated drug, since, in comparison to liposomes, they are more stable as drug delivery formulations with an additional structural element for drug release control, whereas, in comparison to nanogels, lipobeads could provide a higher level of biocompatibility and bioavailability.

Second, superior properties of lipobeads such as higher encapsulation efficiencies, loading capacities, stability on storage and in the bloodstream, a slower and more gradual (more sustainable) drug release profiles for both high molecular weight (e.g., proteins) and small molecule (e.g., doxorubicin) drugs have already been proven experimentally *in vitro*. In animal experiment, the lipobead-delivered combination therapy demonstrated no toxicity on intravenously administered mice, accumulation of drug-loaded lipobeads both in the area surrounding tumor as well as within the tumor itself with an evident payload in the interstitial spaces between tumor cells outside the vasculature, a high therapeutic activity at the targeted site, a significantly greater reduction in both tumor growth rate and tumor mass, and as a result, a drastically increased survival.

Third, the bi-compartmental structure of lipobeads could provide a number of novel and unique options such as a consecutive multistep triggering, i.e., an ability to change the concentration profile of a released drug from burst release and/or pulsatile release to sustained or controlled release, new schemes of drug release (“sponge-like”, “poration”, and “burst”), and combined drug delivery systems based on their reversible and irreversible aggregation.

Fourth, the data reviewed demonstrate the feasibility of the technological platform for drug delivery using lipobeads. To the great extent, the major methods for lipobeads' synthesis (polymerization within liposomal interior and liposome/hydrogel mixing) and for drug loading (polymerization in the course of hydrogel core preparation and soaking the dry hydrogel particles in a drug-dissolved solution) are analogous to those of conventional liposomes and nanogels. Therefore, one can expect that additional technological expenses on the increased complexity of lipobeads production will not be a high cost for the discovered advantages of their use.

Finally, the transition to the next level of complexity, lipobeads, puts forth the ideas on the conceptually new drug delivery systems such as proteo-lipobeads, vesobeads and their combinations, as well as on new mechanisms of lipobead internalization into the cell and drug

release regulated by specific signaling, and allows one to consider the lipobeads as the beginning of Era of the so-called Bioscopic Drug Delivery [1], i.e. the bio-controlled delivery systems for biomolecular drugs. But this will be a topic for another story.

Acknowledgements

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