Syntheses and biomedical applications of hollow micro-/nano-spheres with large-through-holes

Yinsong Si, Min Chen* and Limin Wu*

Hollow micro-/nano-spheres with large-through-holes in shells (denoted as HMLS) have demonstrated great potential in biomedical applications owing to the combination of hollow structure and their porous shells. In this review, we provide a comprehensive overview of synthesis methods of HMLS obtained from the template-directed approach, shell-breaking method, Ostwald ripening and galvanic replacement primarily based on the formation mechanism of the large-through-holes in the shell. We further discuss the biomedical applications of HMLS including guest adsorption and encapsulation of proteins, drug/gene delivery, biomedical imaging, and theranostics. We conclude this review with some perspectives on the future research and development of the HMLS with desired morphologies and properties.

1. Introduction

The past few decades have witnessed great advances in the syntheses and applications of hollow microspheres due to their large surface area, low density, high loading capacity, controllable structure and wide range of potentially applications.1,2 Up to now, tremendous efforts have been made in fabrication of hollow microspheres with controlled composition, tailored structure, and unique properties via various procedures including hard-templatting, soft-templating, and template-free methods.3–6 These synthesized hollow microspheres demonstrate great advantages over solid spheres in catalysis, energy storage and conversion, photonics, and biomedical applications.7–11 As a kind of new hollow material, hollow microspheres with a mesoporous shell have attracted great attention due to their large specific surface area, well-defined ordered mesostructure and tunable pore size, which have shown great advantages in biomedical applications.12 For example, mesoporous silica

---

Yinsong Si received his Bachelor's degree from Zhongyuan University of Technology in 2008 and Master's degree from Donghua University in 2015. Currently, he is a PhD candidate in Fudan University under the supervision of Professor Min Chen. His research interest mainly focuses on the development of multifunctional nanomaterials for biomedical applications.

Min Chen received her PhD degree from Fudan University under the supervision of Professor Limin Wu in 2006. Her dissertation was chosen as one of the “Top 100 National Excellent Doctoral Dissertations” in 2008. Just after finishing the doctorate work, she joined the Department of Materials Science, Fudan University, and was successively awarded “Excellent Young Scientist Foundation of NSFC”, “Shanghai Rising-Star on Science and Technology” and “Shanghai Shuguang Scholar” by Shanghai municipality. She is currently a full Professor. Her research interests include the synthesis, characterization, assembly and properties of novel organic–inorganic hybrid nanostructured materials and hollow spheres.
hollow spheres used as carriers of doxorubicin (DOX) have owned significant merits in cancer therapy.\textsuperscript{13} The large specific surface area of both exterior and interior provides more adsorption or reaction sites for functional substances than that of solid microspheres.\textsuperscript{14} The relatively large voids owing to the hollow structure increase the amount of substances encapsulated while protecting them from damaging or decomposition.\textsuperscript{15} In this way, the cytotoxicity caused by carriers was also depressed to some extent since the amount of carrier materials introduced into body was decreased relatively.\textsuperscript{16} Also the mesoporous pores in the shell provide the channels for the functional substances to be encapsulated into the microspheres and/or controlled released by pH-, light-, biological-, ultrasound-, and thermal-induced stimulation in the subsequent process.\textsuperscript{17–20} Moreover, multifunctional platforms can be further achieved through integrating above two of the functions such as drug delivery, imaging, targeting and/or others by regulating the compositions or structures or by surface modification.\textsuperscript{21–24} Hence, hollow-structured mesoporous microspheres composed of various compositions including polymers, inorganics or hybrids have been largely reported in biomedical applications.\textsuperscript{25,26}

However, recent researches have revealed the size of pores in the shell showing a big difference in the encapsulation and delivery of functional substances.\textsuperscript{27,28} It was found that the hollow spheres with large-through-holes above 10 nm are highly desired for applications involving biomacromolecules.\textsuperscript{29,30} Such HMLS usually have no limitations in proteins or DNA encapsulation or diffusion. While in the case of small pores, typically 2–5 nm for mesoporous hollow spheres, the diffusion becomes slow or even impossible for large sized proteins, which is not efficient for the applications that require fast loading and releasing characteristics.\textsuperscript{31–34} In fact, the structure of large-through-holes in the shell is unique that it has distinguished HMLS from conventional hollow microspheres with no/small pores in the shell due to their special functions in biomedical applications. Thus, more interest in hollow spheres has been transferred to the synthesis and bioapplications of HMLS recently. Various kinds of HMLS with single,\textsuperscript{35,36} multiple,\textsuperscript{37} and hierarchal\textsuperscript{38} holes in the shell, with the morphology of bowl-like,\textsuperscript{19} mouth-like,\textsuperscript{40} golf ball-like,\textsuperscript{41} and cage-like\textsuperscript{42} have been successfully synthesized.

Although several excellent reviews about hollow spheres or hollow mesoporous microspheres have been published recently,\textsuperscript{1,3,9,12,13,43–45} there is no review involving the syntheses and biomedical applications of HMLS to the best of our knowledge. In this review, we will first discuss the synthetic methods of hollow microspheres with large pore size (>10 nm) in the shell based on template-directed, shell-breaking, Ostwald ripening and galvanic replacement reaction approaches. For each method, we provide a critical comment based on our knowledge and related research experience. Then we will present a broad range of biomedical applications of HMLS including the guest encapsulation and immobilization of proteins, drug/gene delivery, biomedical imaging and HMLS-based theranostics. Finally, we conclude this review with some perspectives on further research and development of this kind of hollow micro-/nano-spheres.

2. Synthetic methods of HMLS

2.1 Template-directed approach

The template-directed method is the one of the most widely used strategies, in which hierarchal templates usually composed of two kinds of spheres or nanoparticles with different sizes are used to form the hollow void and large-through-pores in the shell. For example, a composite sphere with a large positively charged polystyrene sphere (PS) with several small negatively charged spheres adsorbed on the surface via electrostatic interactions can be used as a template as a whole. After the hydrolysis and condensation reactions of the precursors on the hierarchal templates and subsequent formation of core/shell structures, the hierarchal templates are removed by either calcinations or chemical etching with acid, alkaline, or organic solvents to form HMLS.\textsuperscript{46}

As shown in Fig. 1, Landon et al. synthesized Au HMLS via a typical template-directed method.\textsuperscript{41} Firstly, a hierarchical template consisting of 100 nm PS beads attached to a 1000 nm silica core was formed by electrostatic attraction between carboxylate-modified PS spheres and poly(diallyldimethylammonium chloride) (PDDA)-functionalized silica. After that, gold seeds were attached to the hierarchal templates by mixing the template solution and gold seed solution together under vigorously stirring. Then an interconnected gold shell was formed through an electroless plating process during which the gold ion was reduced to gold atoms by formaldehyde and the gold atoms preferentially accumulate on the gold seeds. Particles (I) with 100 nm pits were created when the 100 nm PS satellites were removed by dissolution of PS in N,N-dimethyl formamide. Particles (II) were produced by the subsequent removal of the silica core in hydrogen fluoride solution.

The preparation process consists of three major parts: template synthesis, gold plating and template dissolution, which is a
Fig. 1 Hierarchical template scheme used in the synthesis of gold golf balls and hollow gold golf balls. (a) Silica core (gray) functionalized with PDDA and smaller PS satellite spheres electrostatically attached. (b) Nanoparticulated colloidal gold (red) selectively attached onto the PDDA-functionalized silica core (gold seeding). (c) Electroless plating process grows the nanoparticles gold seeds into an interconnected gold shell. (d) Dissolution of the PS satellites completes the synthesis of the gold golf ball particles. (e) Subsequent dissolution of the silica core completes the synthesis of the hollow gold golf ball particles. Reprinted with permission from ref. 41. Copyright 2014 American Chemical Society.

Fig. 2 (a–c) SEM and TEM images of the porous hollow silica spheres after calcination from the templates with different size ratios: (a) 540 nm/280 nm, (b) 540 nm/1100 nm and (c) 260 nm/1100 nm. Reprinted with permission from ref. 47. Copyright 2014 American Chemical Society.

Fig. 3 Schematic illustration of polymer microsphere formation with a hollow core/porous shell structure. Reprinted with permission from ref. 46. Copyright 2005 American Chemical Society.
basic conditions. Subsequent growth of silica seeds to nanoparticles with a size of ~40 nm leads to the coalescence of the adjacent particles. The uncovered areas on PS surfaces were converted into macroporous through holes after core removal through either dissolution or calcination, thus forming the macroporous silica hollow structures with large through holes (size >50 nm) in their shells. Similar structured ZnO hollow spheres with partial open gaps were obtained by Ge et al., which comprised of a mass of nanoparticles.50

A similar strategy but different reaction system was adopted by Zhao et al., who proposed a polymer–metal ion template model, in which the poly(ethylene oxide) interacted with Cu2+ ions forming templates for the preparation of hollow Cu2O crystals.51 Various shaped Cu2O hollow structures, including porous spheres, peanuts, and ellipsoids with different sizes, were obtained by regulating the Cu2+ concentration and pH value of the solution. Furthermore, the templates were not necessarily sacrificed but could be used reversibly. Du et al. fabricated hollow silica HMLS using a thermosensitive polymer, i.e. poly(N-isopropylacrylamide) (PNIPAm), as a reversible template without further calcination or chemical etching.52 PNIPAm chains can reversibly form aggregates or dissolve in aqueous solution by simply regulating the solution temperature with respect to the lower critical solution temperature (LCST). The PNIPam chains behave as soft templates for the formation of core–shell silica nanospheres at elevated temperature (>LCST), and they will then diffuse out of the cores at lower temperature (<LCST), leading to the formation of hollow silica nanospheres.

In fact, some hierarchal templates could even be formed in situ due to phase separation. Minami et al. synthesized polystyrene/polydivinylbenzene (PS/PDVB) hollow spheres with single holes by the self-assembling of the phase separated polymer method, which was a seeded polymerization process assisted by emulsifier sodium dodecyl sulfate.53 As they claimed, it seemed that the hole in the shell was formed because the emulsifier adsorbed at the interface of the droplet affected the self-assembly of PDVB molecules at the interface. In another similar strategy, HMLS were prepared with the assistance of the oil-in-water (O/W) emulsion droplet where a water-soluble polymeric surfactant and an oil-soluble surfactant are competitively adsorbed.54 The shell formed on the water-soluble polymeric surfactant; meanwhile, holes appeared where the oil-soluble surfactant molecules assembled as the shells were formed. Apart from the template-directed method illustrated above, a novel “textured isomorphic synthesis” in highly viscous solutions was introduced to create hollow micro- and nanostructures with defined surface topographies featuring either dimples or holes.55 However, the fabrication process relies on high-aspect ratio nanowires oriented in the vertical direction, which maybe have difficulty in scale-up production of such HMLS.

From the above one can see that the hollow structure and size of the large-through-hole can be well controlled to a certain extent by using the hierarchical templates. However, these template-directed methods are usually multisteps and cumbersome somewhat, even though HMLS with relatively narrow size distribution can be obtained. Meanwhile, the fabrication process is complex, especially a hierarchal template consisting of different sized particles has to be prepared and surface modified in advance, which makes it time- and energy-consuming. Moreover, tedious treatments, such as rinsing, calcination, or chemical etching are indispensable to remove the template in the following step, in which the structure of the shell or the monodispersity of HMLS may be damaged, hampering their biomedical applications.

### 2.2 Shell-breaking method

The shell-breaking method is termed by the formation mechanism of the large-through-hole in the shell. In this method, the holes of HMLS can be created by inside-out diffusion or evaporation of solvents,56 soluble polymers,57 monomers,58 oligomers59 and precursors60 through the shells, or by the fracture of the shells due to their shrinkage during the fabrication process,61 or by selective etching owing to the composition/structural difference in the shells.27,62,63 One thing in common is that a certain section in the shell “breaks” and turns out to be a hole. A single large-hole in the shell is mostly formed in this method, while a multipore shell can also be obtained either. This method is based on the template-assisted method to some extent and the hollow structure is formed after removing the template core. However, the formation of holes shows a big difference from that of the template-directed approach, in which both the hollow structure and the holes are formed only after their own templates are removed.

#### 2.2.1 Out-diffusion or evaporation of the internal materials

Using the swelling and fast freezing strategy, Im et al. reported the preparation of HMLS with controllable holes in the surfaces.64 Fig. 5 shows the three major steps involved in the transformation of solid PS beads into HMLS. In the first step, solid PS beads were suspended in water and swollen by adding a good solvent of the polymer (for example, toluene or styrene). The immiscibility between toluene and water drives toluene to diffuse into the beads, expanding their radius. In the next step, the swollen particles were quickly frozen by pouring the aqueous suspension into liquid nitrogen (−210 °C). Because of the poor thermal conductivity of PS, a temperature gradient was formed in the radial direction and solidification of toluene started from the surface. Thus a void inside each bead was created due to the volume shrinkage caused by solidification. In the last step, the frozen sample was slowly warmed up to let toluene evaporate in a vacuum or under ambient pressure.
Once the temperature reached the melting point of toluene (−93 °C), the PS chains would migrate towards the surface of each particle driven by the flux of evaporating toluene, increasing the void size. A hole was formed in the surface of each PS particle because of the evaporation flux of toluene. Later in their work, hollow microspheres with a single large hole and small holes coexisting in the surfaces were obtained by increasing the degree of swelling.65 By using a similar procedure, Yin et al. obtained PS HMLS successfully, while they thought that the structure was caused by polymer/solvent phase separation during freezing and subsequent interfacial free energy minimization.66

Other kinds of polymer HMLS such as PMMA, polycaprolactone (PCL) and poly(ε-lactide) have also been successfully prepared.64 Moreover, controllable holes can be obtained by using different solvents to swell the PS spheres by this swelling and fast freezing method. Recently, Hyun et al. did more detailed work based on this method to regulate the structure of the PS HMLS by controlling the amount of solvent used to swell the polymer beads.19 As shown in Fig. 6, the sizes of the holes were approximately 50, 350, and 600 nm when the toluene-to-water ratio was 0.01, 0.05 and 0.075, respectively, demonstrating the size of the hole had a direct relationship with the ratio. Fig. 6d shows that the hole size \((D_H)\) and volume fraction of solvent \((1 - \phi)\) had similar changes as a function of the solvent ratio, suggesting the formation of holes was caused by the initial flux of evaporating toluene. Then the remaining solvent evaporated through this preferred path (the hole).

These results suggest that this swelling and fast freezing method can be used as a general route to fabricate polymer HMLS with good monodispersity. However, a fast cooling setup and a toluene evaporating temperature of below 0 °C are indispensable in this route. Also the products obtained by this method are limited to the organic polymers that can be prepared as colloidal particles and then be swollen with an appropriate solvent.

A poly(o-toluidine) HMLS with a similar morphology but quite different formation mechanism were obtained by Han et al., in which the hole was formed during the polymerization process.63 Droplets were formed by the o-methoxyaniline monomers themselves in the aqueous solution owing to the amphiphilic structure of the monomer. After being initiated by adding ammonium persulfate (APS), the polymerization took place at the water/droplet interfaces because of the hydrophilicity of APS, which resulted in the formation of original hollow nanospheres. As they claimed, with the consumption of monomers in the outer surfaces of hollow spheres, monomers diffused from the droplets to the external surfaces to continue the polymerization reaction. Hence, it was believed that the diffusion flux of monomers caused the formation of the hole in the shell of each hollow microsphere. Based on this work, they also demonstrated a facile and environmentally friendly approach for the fabrication of poly(o-methoxyaniline) HMLS by chemical polymerization in the absence of acid.67 As shown in Fig. 7, when the concentration of the monomer decreased from 0.10 to 0.02 M, the size of the hollow spheres decreased from 3000 nm to 700 nm and the size of the hole decreased from 800 nm to 200 nm, respectively. However, it was also found that the polymer hollow spheres often fused together when the concentration of the monomer was relatively low, for example, 0.02 M. Hence, synthesis of monodisperse hollow spheres with a small size by this method is still challenging.
To explore functionality of the inner surfaces of artificial hollow spheres, Mandal et al. fabricated an open-mouthed metallic (platinum, Pt) microspheres (Fig. 8C and D) with sufficient accessibility at both interior and exterior surfaces.40 These open-mouthed Pt microspheres were prepared by using PS spheres (Fig. 8A) as templates for platinum coating (Fig. 8B). After formation of the capsule shell composed of fused crystalline nanoparticles, calcination to remove the PS template induced the fusion between Pt nanoparticles, leading to shells free of small pores. Due to the escaping residues of the decomposing template, mouth-like openings were probably formed. TEM (Fig. 8E) confirmed the hierarchic structure of the hollow capsules composed of Pt nanoparticles, which were homogeneously distributed in the open-mouthed microcapsules (Fig. 8F).

It is especially important to note although only one hole formed in the shell via the above method, precise size control of the hole seems to be easy for this kind of HMLS. For instance, Li et al. fabricated polyurethane (PU) hollow microspheres with size tunable single holes in their shells through a facile self-assembly diffusion process (Fig. 9).57 Firstly, the polyethylene glycol modified PU polymers were completely dissolved in chloroform. By adding 10 times the volume of water, the PU polymers were subsequently self-assembled into micelles in which the chloroform was encapsulated. But the chloroform droplets encapsulated were not exactly at the center of the PU micelles. Hence, when the PU micelles were then transferred to methanol solution to allow the diffusion of chloroform, the primary holes were formed at the thinnest part of the micelle walls, where the chloroform was preferred to diffuse through. Since the chloroform could gradually dissolve the PU polymers surrounding the holes, the primary holes became larger and larger with increasing diffusion time. As a result, the sizes of holes could be tuned from 75 to 210 nm by adjusting the diffusion time from 5 to 20 h.

In the shell-breaking strategy, multiporous hollow microspheres have also been fabricated by some researchers. Fujiwara et al. synthesized silica hollow spheres with nano-macroholes (>100 nm) in the shell by adding some water-soluble polymers to a solution of the water/oil/water double emulsion system.68 As shown in Fig. 10, firstly, they added water-soluble polymers (sodium polymethacrylate) to sodium silicate solution as the inner water phase (IWP). A W/O emulsion of this IWP with an oil phase of \( n \)-hexane solution (including Tween 80 and Span 80) was mixed to the outer water phase with \( \text{NH}_4 \text{HCO}_3 \), leading to the formation of a W/O/W emulsion system. After a few minutes, silica hollow particles precipitated in the mixed solution. They considered that the additive polymers permeated the shell of the particles since no polymers were found in the synthesized silica particles. The passage of the additive polymers prevented the formation of the silica shell matrix in the domains where the polymers passed through. Finally those domains became macroholes in the shell of silica hollow particles. Because the manners of the passage depended on the additive polymers, the macrohole structures varied with different polymers employed.
Fabrication of multifunctional HMLS is also very important since they are expected for some special applications like magnetic resonance imaging (MRI), and other bio-related applications. Li et al. synthesized cage-like silica hollow spheres loaded with superparamagnetic iron oxide nanoparticles (SPIONs) incorporated into their macroporous shells through a one-step oil-in-decalin monomer (DEG) microemulsion route (Fig. 11).\(^{42}\) The oil phase containing a mixture of TEOS, toluene, and SPIONs, was emulsified by a nonionic amphiphilic surfactant Triton X-100 in a surrounding hydrophilic DEG environment. Ammonium hydroxide was added subsequently to initiate the hydrolysis and condensation reaction of TEOS. Though the toluene used in the oil phase did not participate in any action, however, the entrapment of liquid toluene was believed to be the cause of the formation of such macroporous hollow structures. The density increased with the transformation from liquid TEOS to solid silica at the interface of oil droplets and DEG, which led to the contraction of the oil drop size. As the shells continued to shrink, toluene erupted from the shell surface. This is how the nano-macro-sized through holes in the shell were formed. As the amount of toluene was increased, toluene became a liquid “template” instead of small toluene “bubbles” during the silica formation process and hollow spheres were formed with the aid of these liquid templates.

It is worth noting that holes with wide distribution in the shells were formed with increasing SPION concentration. Hence, it seems that the mechanism would be perfect if one more experiment was done by using no SPIONs while maintaining the amount of toluene high to be checked whether the SPIONs made a combined or whole contribution to the formation of the holes in the shells.

By using a similar strategy, Chang et al. prepared single-holed poly methylsilsesquioxane (PMSQ) hollow spheres via a solvent evaporation route using coaxial electrohydrodynamic atomization.\(^{69}\) PMSQ was used as the model shell material to encapsulate a volatile liquid perfluorohexane as the core. Hollow microspheres were subsequently formed after the evaporation of the liquid core. By regulating the processing parameters, the diameters of both the microspheres and the pore could be precisely controlled.

**2.2.2 Shrinkage of the shell.** Among the shell-breaking methods, shrinkage of the shell can also result in the formation of HMLS. For instance, Guan et al. reported a straightforward method to synthesize single-holed core-shell hollow polymer microspheres by a consecutive two-step procedure including pre-polymerization and polymerization/cross linkage at the surface of carboxyl-capping PS beads.\(^{70}\) Subsequently, the hollow microspheres with a single large hole were obtained by the dissolution of the PS cores with tetrahydrofuran (Fig. 12).

The formation of the single hole in the shells was resulted initially from the voids which were formed and expended due to the different molecular weights or cross-linking densities in the prepolymerization and subsequent polymerization stage. Successive volume shrinkage of the original layer of the prepolymer on the surface of the PS beads finally resulted in the fracture of the outer film on the top of the hole. That is how the single hole core/shell microspheres were formed, as shown in Fig. 13.

**2.2.3 Composition/structural difference-based selective etching.** HMLS can be also formed when some biomolecules are treated by a carbonation method. Ni et al. presented a facile method for fabricating porous hollow carbonaceous spheres (PHCSs) with controllable meso- and macroporous shells through mild hydrothermal treatment (180–200 °C) of S. cerevisiae cell.\(^{27}\)

The as-obtained microspheres endowed with amphiphilic properties due to the coexistence of hydrophilic and hydrophobic groups on the surfaces of PHCSs. Glutaraldehyde (GA) was added to enhance the strength of the polysaccharide networks and thus integral hollow microspheres could be obtained. The average size of the pores in the shell could be tuned from below 5 nm (Fig. 14a and b) to 43 nm (Fig. 14c and d), by varying the concentration of GA aqueous solution. Further acid etching could increase the pore size to 50 nm (Fig. 14e and f),
even macropores with size exceeding 100 nm could be seen clearly in some samples.

The formation of meso- and macropores on the shell was attributed to the different hydrolysis ability of the amorphous matrix and fibrillar network existed in the cell-wall polysaccharide networks (CWPNs), and the hydrolysis resistance of CWPNs enhanced by GA. Through covalent binding with glucose residues of adjacent glucan chains, GA molecules could firmly link the glucan chains of CWPNs. Firstly, some small hydrolysis channels appeared in the shells. Then, individual hydrolysis channels kept enlarging and incorporating with each other during further hydrothermal treatment. Finally, penetrable meso- and macro-pores emerged in the microsphere shells.

Besides biomolecules, artificial hollow mesoporous nanospheres can also be selectively etched utilizing the reversible chemical bonding/debonding characteristic during the hydrolysis and condensation process. Hollow mesoporous silica nanoparticles (HMSNs) are of great significance in drug/gene delivery systems. Although HMSNs with small mesopores (2–3 nm) in the shell have been reported extensively and showed excellent drug-delivery systems both in vitro and in vivo, hollow silica nanoparticles (HSNs) with well-defined large pores (>10 nm) in the shells have been fabricated by tuning pore sizes of the small pore-sized HMSNs to encapsulate and deliver large entities, such as siRNA or DNA. The small pore-sized HMSNs were prepared by selectively etching the solid core based on the difference in condensation/densification degrees between the core and the shell of the solid silica core/mesoporous silica shell nanoparticles (denoted as sSiO$_2$@mSiO$_2$), which were synthesized by the co-condensation of TEOS and octadecyltrimethoxysilane (C$_{18}$TMS) on the surface of Stöber-based silica nanoparticles. As illustrated in Fig. 15, due to the reversible chemical bonding/debonding during alkoxide hydrolysis and condensation under alkaline conditions, the pore sizes could be enlarged beyond 10 nm by Si–O bond breakage or could be reduced to zero by the Si–O bond reformation.

HMLS with single hole in the surface is mostly obtained by the shell-breaking route, although hollow microspheres with multi-holes could also be obtained in some cases. The outflow or evaporation of the internal materials, the shrinkage of shell,
and etching specific parts/nanopores in the shells have been used to prepare the HMLS. It is worth noting that the hole in the shells can play a key role in adsorption and encapsulation of various sized functional guests. Moreover, the holes can also be sealed in post-processing such as thermal treatment, further precipitation of some precursors, or capping.

2.3 Ostwald ripening process

Recently, the Ostwald ripening process has been proposed as a template-free strategy to fabricate hollow and porous microspheres, which has attracted much attention owing to the potential in fabricating HMLS in a convenient way.\(^76,77\) In the theory of Ostwald ripening, large particles grow at the expense of smaller ones, since they are more energetically favored than small ones.\(^78\) Here we just emphasized the formation of the large-through-hole in the shell of hollow microspheres since a detailed account of the Ostwald ripening method to fabricate hollow inorganic spheres has been presented in another review.\(^3\)

Shiomi et al. reported a cage-like hollow spherical silica (CHS, Fig. 16) with a through-hole (50–250 nm diameter) structure by calcination of lysozyme–silica hybrid hollow particles (L–SHHs), which were synthesized by the combination of sonochemical treatment and the silica precipitation activity of lysozyme.\(^79,80\) It was found that lysozyme molecules were uniformly dispersed within the silica matrix.\(^81\) They claimed that the synthesis route to CHS was somewhat different from the transcription method using a bioorganic polymer just as a template, because the through-hole sizes of the CHS synthesized were 10 times larger than the molecular size of lysozyme. The shape of the through holes formed after calcination of lysozyme–silica hybrid was dependent on neither the shape of the lysozyme molecule nor that of its aggregation forms. Upon comprehensive analysis of TGA and pore size distributions, they concluded that the formation process of CHS during calcination was comprised of two steps: the removal of lysozyme resulting in the formation of mesopores by the templating effect of lysozyme (first step), and the subsequent restructuring of the silica network leading to a morphological change to the CHS (second step).

Following this work, a cagelike hollow aluminosilicate (CHA) with vermiculate micro-through-holes was obtained by the Al-containing alkaline hydrothermal treatment of lysozyme–silica hybrid particles.\(^82\) The formation of CHAs with vermiculate micro-through-holes is thought to occur through Ostwald ripening. Fig. 17 exhibits the formation process of the CHA. Firstly, lysozyme molecules were dissolved out of the silica matrix of the hybrid structure, due to the easy degradation of the peptide bond of the protein by the alkaline solution. Simultaneously, the formation of aluminosilicate gel from silica and aluminate solution occurred in the presence of alkaline cations.\(^83\) The aluminosilicate gel would be prone to formation in the surface silica regions rather than the inner silica regions. The penetration of aluminate solution to the inside of the preCHA structure might be delayed by the presence of lysozyme or its degradation products. Moreover, the curvature of the inner silica regions after removing lysozyme would be relatively higher than that of the surface regions. According to the theory of Ostwald ripening, the relatively large particles continue to...
grow spontaneously consuming the relatively small particles, since the former is more energetically favored than the latter.\textsuperscript{54} Hence, the aluminosilicate gel on the surface grew into a shell structure and preserved its initial shape, whereas any small nanoparticle aggregations formed inside were gradually dissolved and disappeared. The initial through-hole structure gradually changed to a more distinct vermiculate shape. The growth of the shell structure continued until the supply of aluminosilicate aggregates inside the pre-CHA material runs out. It is obvious that the tedious template preparation process can be avoided in this method.

Ostwald ripening is a simple route to synthesize HMLS without employing either template or surfactant. Using this strategy, CuInS\(_2\), ZnO/ZnCo\(_2\)O\(_4\), and FeMoO\(_4\) hollow spheres with large-through-holes in the shell were also obtained.\textsuperscript{85-87} Moreover, bifunctional highly fluorescent hollow BaMoO\(_3\):Pr\(^{3+}\) microspheres with a large amount of pores (17.5 nm) were successfully synthesized via the Ostwald ripening strategy, which exhibiting a great promise for drug delivery due to their combination of excellent luminescent properties and a hollow porous nanostructure.\textsuperscript{88} In another case, high-yield ternary ZnO/ZnS/\(\gamma\)-FeO\(_3\) hollow spheres with an average size of 600 nm were prepared rapidly and the reaction period was as short as 30 min.\textsuperscript{89} Similar structured hierarchical ZnO hollow spheres (400–500 nm in diameter) consisting of ZnO nanoparticles with a diameter of approximately 15 nm was obtained by He et al. via this simple, rapid (<1 h), and template-free method.\textsuperscript{90} Therefore, it can be concluded that the Ostwald ripening strategy have displayed great advantages of facility. However, to date, this strategy has only focused on some special inorganic compounds.\textsuperscript{3} The diameters of synthesized microspheres were micron-sized and inhomogenous in some cases, which may limit their applications in biomedical application. Moreover, the large-through-holes in the shell are less controllable compared with the template-directed approach. Hence, precise control of the morphology and structure is needed in further research.

2.4 Galvanic replacement reaction

Except the methods mentioned above, galvanic replacement reaction is another route to fabricate HMLS. Xia et al. first described the galvanic replacement reaction for the generation of nanoscale hollow noble metal structures with well-defined void spaces and homogeneous, highly crystalline walls, which laid a foundation for the fabrication of metal HMLS.\textsuperscript{91,92} In this reaction, a metal nanostructures (suspended in solution) with lower reduction potential was added to a salt solution of another metal with higher reduction potential. Due to the difference in reduction potentials, Au ions in the salt solution were reduced on the surfaces of the Ag nanoparticles which simultaneously oxidized. Then a thin, incomplete alloy shell with pinholes formed (Fig. 18B). As the reaction continued, the net mass flowed toward the shell from the central region of the nanoparticle because of the Kirkendall effect. The shell then reshaped and resulted in the reduction in the sizes of the pinholes through Ostwald ripening (Fig. 18C). Due to the presence of Ag in the nanoparticle at this stage, many holes formed in the following dealloying process (Fig. 18D), in which Ag was oxidized by H\(_2\)O\(_2\), Fe\(^{3+}\) and ammonia.\textsuperscript{96-98} Smaller components consisting of pure Au were formed when Ag was fully oxidized (Fig. 18E). However, as they claimed, the residual Ag core had a negative influence in the stability and toxicity of HGNs, which should be carefully studied prior to any bioapplications in vivo.

Lu et al. synthesized a novel kind of cobalt yolk/gold shell nanospheres with a cavity by a one-step galvanic replacement reaction first, and then uniform spherical gold nanocages were obtained after strong acid etching of these obtained yolk/shell nanocomposites (Fig. 19).\textsuperscript{99} Park et al. also synthesized hollow gold nanoparticles (HGNPs) based on the galvanic replacement reaction method in which colloidal silver nanoparticles were synthesized first and then used as templates for the reduction of gold ions in the HAuCl\(_4\) solution.\textsuperscript{91,93,100-103} To use in vivo, the obtained HGNPs were coated with a layer of polyethylene glycol monomethyl ether thiol through a thiol bond.\textsuperscript{104} The pegylated HGNPs with an average diameter of 52 nm in this study have showed the highest cellular uptake.\textsuperscript{105,106} Fig. 20A reveals the morphology of pegylated HGNPs and large through-holes could be seen in the shell, which had a small size and narrow distribution (Fig. 20B).

\begin{equation}
3\text{Ag(s)} + \text{AuCl}_4^- (\text{aq}) \rightarrow \text{Au(s)} + 3\text{Ag}^+ (\text{aq}) + 4\text{Cl}^- (\text{aq})
\end{equation}

The produced gold is confined to the vicinity of the silver nanostructure surface, then nucleates and grows into very small particles, eventually evolving into a thin shell around the silver template.\textsuperscript{93} Depending on the shape of the templates, various hollow metal nanostructures such as hollow nanospheres, nanocubes, nanotubes could be obtained by galvanic replacement reaction.

Gold HMLS could also be obtained in the galvanic replacement reaction.\textsuperscript{93,94} Goodman et al. synthesized hollow Au nanoshells (HGNs) with sacrificial Ag cores through a galvanic replacement reaction in which a salt solution of Au was added to the colloidal solution of Ag.\textsuperscript{95} Due to the difference in reduction potentials, Au ions in the salt solution were reduced on the surfaces of the Ag nanoparticles which simultaneously oxidized. The produced gold is confined to the vicinity of the silver nanostructure surface, then nucleates and grows into very small particles, eventually evolving into a thin shell around the silver template.\textsuperscript{93} Depending on the shape of the templates, various hollow metal nanostructures such as hollow nanospheres, nanocubes, nanotubes could be obtained by galvanic replacement reaction.
It is noteworthy that high colloidal stability was maintained for almost 2 months by measuring the size (Fig. 20C and D), which was increased by approximately 10% and 25% after 50 and 60 days, respectively.

The galvanic replacement reaction can be employed as a general route to fabricate hollow structures with various shapes and sizes for metallic hollow structures. Moreover, the porosity can be controlled through modifying the synthesis process. It is noteworthy that a metal HMLS with size of several decades of nanometers or even smaller can be easily obtained in this method, which is greatly desired in drug delivery systems. However, the galvanic replacement reaction has the same problem as Ostwald ripening that the holes are less controllable compared with the template-directed approach. And the as-prepared HMLS are limited to noble metals, transition metal oxides or sulfides. Furthermore, the residual starting material in the HMLS may cause severe damage to cells and thus the reaction degree needs to be finely controlled, and strict tests are also needed prior to any usage in biomedical applications.

3. Biomedical application of HMLS

Owing to the high specific surface area, huge hollow cavity, and the large pores in the shell providing the channels for the functional substances to be encapsulated into the microspheres and/or controlled released, HMLS have attracted much attention in various fields especially in biomedical applications, such as guest encapsulation and immobilization of proteins, drug/gene delivery, biomedical imaging and theranostics. Due to the limit of length, the research studies on HMLS-based biosensors and bio-catalysis are not discussed here.

It is noteworthy that the size of HMLS should be paid special attention since they have to penetrate the vessel walls and tissue matrices and uptake by target cells in *in vivo* experiment and clinical applications. The cell uptake process, *i.e.* endocytosis, is realized by four major pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, pinocytosis, and phagocytosis. Large particles are internalized by pinocytosis and phagocytosis, while small particles are mainly internalized by clathrin- and caveolae-mediated pathways. Generally, nanoparticles demonstrate higher intracellular uptake than microparticles. Even though nanoparticles may facilitate the cellular entry process, they seem to have no size limit up to 5 μm to gain cellular internalization. Amidon *et al.* found that nanoparticles (100 nm) had a 2.5 and 6 times greater cell uptake than microparticles of 1 μm and 10 μm in size, respectively. This trend was further verified by other researches. All of the size, surface properties, shape of the cargos and the type and physiological condition of the cells can affect the cellular uptake efficiency. The parameters influencing the endocytosis process have been deeply discussed in several other reviews. In drug delivery and imaging *in vivo*, HMLS usually penetrate the vessel walls and are uptaken by tumor cells. In gene delivery, HMLS with gene can be either uptaken by tumor cells or by the macrophages to initiate immune response. However, it can be seen that some HMLS mentioned in this review are of microscale and have no advantages in applications *in vivo*. Thus, HMLS with optimized morphology are expected in future studies, even better with proper composition and surface properties to reduce cytotoxicity and improve biocompatibility simultaneously.

### 3.1 Guest encapsulation and immobilization of proteins

The above mentioned PS HMLS synthesized by Im *et al.* have been used to load functional materials directly, such as, coumarin-6 and dinitrophenol-conjugated bovine serum albumin (DNP-BSA), which were used as the samples of small molecules and...
macromolecules for encapsulation, respectively.\textsuperscript{64} After loading, the holes could be closed by annealing the system at a template slightly above the glass-transition temperature of PS. Both the small molecular coumarin-6 (Fig. 21a) and the macromolecule DNP-BSA (Fig. 21b) could be encapsulated in the PS HMLS. Moreover, the superparamagnetic iron oxide nanoparticles were also successfully encapsulated in the PS particles by a similar process (Fig. 21c).

The hollow cavity can be used as a nanoreactor besides loading of functional materials. As mentioned earlier, Shiomi et al. synthesized a cagelike hollow aluminosilicate (CHA) with vermiculate micro-through-holes in the shells via an Ostwald effecting method.\textsuperscript{82} Then they demonstrated the good permeability of biomacromolecules due to the micro-through-holes and the inorganic shell of CHA by using a “ship-in-bottle” strategy (Fig. 22). They chose the Discosoma coral (DsRed), a red fluorescent protein with a height of 4 nm, as a model protein, which is thought to be sufficiently small to penetrate the micro-through-holes whose diameters reach tens of nanometers. After diffusion of the DsRed monomer into CHA, polymeric DsRed was formed by crosslinking each monomer with GA. The DsRed molecules were successfully encapsulated within the CHA since fluorescence inside the CHA was clearly observed. As a comparison, solid lysozyme–silica hybrid particles without any through-hole and inner space were also tried, which proved that DsRed was localized only on the surfaces of particles by physical adsorption.

Another adsorption test with BSA clearly showed that larger pores could greatly enhance the protein loading. For PHCS-I (mesopores <5 nm in the shell) and PHCS-III (macropores about 50 nm) mentioned earlier\textsuperscript{27} the protein loadings were 2.4 and 22.0 mg g\textsuperscript{-1}, respectively. For PHCS-I, the adsorption of proteins occurred mainly on the surfaces of the microspheres and no proteins in the interior cavities could be observed (Fig. 23a). By contrast, for PHCS-III, plenty of proteins in the interior voids could be observed (Fig. 23b). Moreover, the rapid penetration process was almost complete within 15 min. It is worth noting that the encapsulation was rather stable since it could bear drastic shaking and centrifugal separation during the washing cycles (Fig. 23c). It is quite interesting that the encapsulated BSA could also be released in a controlled manner although rather stable under aqueous conditions. In the \textit{in vitro} release test, about 17.9 and 88.2\% of the loaded BSA could be released into the aqueous medium when the pH value of the medium was around 7.0 and 9.0, while no BSA release was observed when the pH was 4.8.

HMLS composed of various compositions including polymers, inorganics or hybrid have been also studied due to their multifunctions such as drug delivery, imaging, targeting and/or others by regulating the composition and structures or by surface modification. For instance, based on the synthesis of hollow poly(3,4-ethylenedioxythiophene) (PEDOT) particles with single holes, Luo et al. presented the first example of composite HMLS,
which was consisted of biocompatible PEDOT and magnetic iron oxide (Fe$_3$O$_4$) nanoparticles.\textsuperscript{130,131} The poly(EDOT-OH) coated PS spheres tended to form poly(EDOT-OH) capsules with single holes after the PS cores were removed by toluene. The magnetic, single-holed poly(EDOT-OH) hollow spheres were employed in the removal of lysozyme, which had a protein of 14.4 kDa with a radius of 2 nm. The lysozymes could be both non-specifically adsorbed on the poly(EDOT-OH) surface and trapped in the hollow spheres. After the adsorption process, the concentrations of the lysozyme remaining in the solution mixed with the poly(EDOT-OH) hollow capsules was reduced to 0.33 mg mL$^{-1}$, which was much lower than that of the original solution (0.50 mg mL$^{-1}$) and that of the solution mixed with the poly(EDOT-OH) coated PS solid beads. Moreover, the results of cell viability experiments indicated that the samples showed low toxicity for both the cells, and the poly(EDOT-OH) coating improved biocompatibility.

Although the above mentioned HMLS have demonstrated excellent adsorption and encapsulation ability to the guest materials, further in vivo tests are necessary in biomedical applications. Some researches proved that HMLS with the hierarchical pore structure can selectively encapsulate functional materials while protecting them from destruction by external substances. Ortac et al. fabricated a dual-porosity hollow nanoparticles for immune protection and delivery of nonhuman enzymes.\textsuperscript{38} Firstly, synthetic hollow mesoporous nanoparticles (SHMS) with a nanoporous (pore size <2 nm) material and a mesoporous (pore size, 5–50 nm) shell simultaneously were synthesized by a typical hard templating method. To fabricate SHMS (Fig. 24A), PS aggregations formed by electrostatically attraction of oppositely charged and different sized PS nanoparticles were used as templates for the silica layer to grow selectively. Once the desired thickness of silica was formed, the template was removed by dissolution or calcination, leaving the silica SHMS. Then SHMS was loaded by diffusion of macromolecules, i.e., enzymes in this study, through their mesopores. Subsequently, the negatively charged SHMS loaded with enzymes were transformed to positive charge by adsorbing poly-L-lysine. Thus TMOS could be used again to grow silica layers on the surfaces and sealed the mesopores of SHMS, converting SHMS to synthetic hollow enzyme loaded nanospheres (SHELS) (Fig. 24B).

This design is so exquisite that some unique benefits can be provided from the special structure of the products. As shown in Fig. 24C, the enzyme is essentially hidden from the immune system because antibodies are too large to pass through the nanopores meanwhile small molecules in the surrounding environment can still interact with the loaded enzyme via diffusion through nanopores. Moreover, SHELS can be further coated with passivating and targeting ligands without any chemical modification of the payload. The test results confirmed that SHELS had a much better protection of the loaded enzyme against proteolysis by antibodies compared with hollow silica nanospheres (SHS), SHMS and sealed SHS under the same conditions. This was contributed to the prevention of antibody access to the enzymes encapsulated within SHELS. The protection ability was quite stable and a long residence time of SHELS in tissue up to 2 months was observed. The different L-asparagine depletion ability of free Elspar and SHELS-Elspar in naive mice and immunized mice (i.e. in the absence/presence of neutralizing antibodies) validated the protected operation of enzymes (Elspar) in therapeutically relevant in vivo setting.

3.2 Drug/gene delivery

3.2.1 Drug delivery. HMLS, especially those with functions such as targeting and controlled release, have showed great advantages over conventional carriers.\textsuperscript{132} Some studies indicated that the intracellular glutathione (GSH) concentration (1–10 mM) is substantially higher than extracellular levels (0.002 mM in plasma); meanwhile, the GSH concentrations in cancer tissues can be many times higher than that in normal tissues.\textsuperscript{133} In addition, lower pH values in tumor cells and tissues have also been found. Utilizing these properties, Wang et al. fabricated a kind of single-holed GSH-responsive degradable hollow silica nanoparticles (G-DHSNs, Fig. 25) by introducing a kind of disulfide bond bridged silane (BTOCD) to the G-DHSNs for efficient intracellular drug delivery.\textsuperscript{134} The large single-hole in the G-DHSNs shell was formed owing to etching of the PS nanosphere core. The synthesized G-DHSN drug delivery system could be decomposed by cleaving the disulfide bond via GSH and pH stimulation simultaneously to release the loaded DOX. The decomposition experiments of the G-DHSNs in response to GSH showed that the hollow feature started partly broken in the early time, and eventually complete fragmentation of the G-DHSNs was found after 48 h, while the morphology of G-DHSNs presented a negligible change.
without GSH. The loading capacity of DOX was relatively high due to the hollow porous structure of G-DHSNs.

Once DOX was loaded and DOX–G-DHSNs were uptaken by cells, the loaded DOX would be released owing to the higher concentration of GSH, and thereby inhibit TCA8113 cell growth. The cell-killing ratio was about 75.5% at the drug concentration of 10.0 μg mL⁻¹. The CLSM results obviously suggested that DOX–G-DHSNs were internalized and DOX was released to reach cell nuclei within 24 h (Fig. 26). Considering its low blood compatibility and noncytotoxicity TCA8113 cells in this study, G-DHSNs could be used as safe and promising drug nanocarriers.

Among various kinds of stimuli-regulated release, temperature has been widely used to trigger the drug release due to the variation of local body temperature in response to surrounding conditions or diseases.¹³⁵ To make a new system for temperature controlled release, Hyun et al. prepared a PS HMLS, which allowed for quick, efficient loading of small molecules, macromolecules, and even nanoparticles at least 50 nm in size due to the large-through-hole in the shell.¹⁹ Furthermore, as shown in Fig. 27a and b, the hole in the shell could be corked with a phase-change material (PCM), which has a melting point of 38–39 °C and capable of reversible, solid–liquid transition in response to temperature variation.¹³⁶ To demonstrate the temperature-sensitive release ability of this PS HMLS, a fluorescent dye (rhodamine B) was encapsulated in the voids of PS HMLS as a probe. After loading of the dye, drying and subsequent sealing of the holes with PCM, the sample was washed to remove dye molecules absorbed on the outer surface. The fluorescence micrograph in Fig. 27d verified successful encapsulation of rhodamine B in the voids of the PS HMLS. Moreover, it could be concluded that the capping was stable enough to prevent the leakage of the loaded dye during washing of the sample. The obvious difference between release profiles at various surrounding temperature (Fig. 27e) demonstrated that the pre-loaded dye molecules could be encapsulated in the HMLS at a temperature below the melting point of the PCM whereas they could be released instantly when the temperature slightly higher than the melting point. Moreover, binary PCMs with different weight ratios (x) of 1-tetradecanol to lauric acid were used to cap the PS HMLS. Compared with the sample consisted of only 1-tetradecanol, a faster release at x = 0.6 was obtained, as shown in Fig. 27f.

Very recently, Li et al. synthesized novel asymmetric hollow silica nanocages (100–240 nm) with a single big hole (~25 nm) and uniform mesopores (2–10 nm) in the shell simultaneously.¹³⁷ This is a very interesting work since the hollow voids (35–170 nm) and big holes can serve as a storage space with a passage for large guests while the mesopores can also serve as a storage space and channels for small molecules. After being further imbued with upconversion nanoparticles (UCNPs), these eccentric single-hole mesoporous nanocages (UCNP@MSN nanorattles) were used as nanocarriers for the dual-sized guest co-delivery system for BSA (21 × 4 × 14 nm³) and DOX (<1 nm³). The loading capacities for BSA and DOX were 342 and 33.6 mg g⁻¹, respectively. As shown in Fig. 28A, the UCNP@MSN nanorattles showed a good biocompatibility since the viability of the cells was maintained to be more than 95% even at a high concentration (~100 μg mL⁻¹). The modification of temperature-sensitive phase change materials (1-tetradecanol) on the outer surface of the UCNP@MSN nanorattles and light sensitive azobenzene (Azo) molecules in the mesoporous shell channels played a key role in controlled release of the dual-sized guests independently (Fig. 28C).
Compared with the free release of dual-sized guests from the nanocarrier without the modification of the switch molecules (Fig. 28B), the independent release of BSA and DOX from switch molecule modified nanocarriers could be finely controlled with/without the stimuli of the switches (Fig. 28D). It is worth noting that, owing to the NIR to UV/vis optical properties of UCNPs, transformation of Azo molecules between the $\text{cis}$-isomer and the $\text{trans}$-isomer under irradiation of UV/vis light resulted in the back and forth wagging motions of Azo molecules, which propelled the release of the encapsulated DOX in the mesopore shells.\textsuperscript{138} As claimed, this discovery of the single-hole mesoporous nanocages may lead to further development of new concepts and architectures of nanocarriers, which contributed a lot to multi-drug delivery and independent release.

### 3.2.2 Gene delivery

Gene therapy has been considered a significant and promising therapeutic option for the treatment of genetically caused diseases including cancer.\textsuperscript{139,140} To deliver DNA or siRNA into the cytoplasm of the target cell efficiently, nanocarriers with small mesopores (2–3 nm) have been used and showed various merits including prevention of degradation, high loading capacity, specific targeting, and controlled release.\textsuperscript{141}

What limited the loading or encapsulation of these biomacromolecules (e.g. DNA, siRNA) is that the pore size of the nanocarriers usually is too small for genes to get through. Thus DNA or siRNA molecules are usually present on the surface of nanocarriers.

The large-pore sized hollow silica nanoparticles (HSNs) mentioned above synthesized by Chen \textit{et al.} have been successfully used as nanocarriers for siRNA delivery due to large pores in the shell. The encapsulation of siRNA into the pores provided the protection of siRNA from degradation by RNAase meanwhile the release channels for the siRNA to get through. Considering its strong escape ability from the endosomes in cells, cationic polyelectrolyte polyethyleneimine (PEI) with different molecular weights was used to modify the inner pore surface of the large-sized pores of HSNs to make it positively charged (Fig. 29).\textsuperscript{142} Thus the positively charged PEI-HSNs could bind negatively charged siRNA through electrostatic attractions. To assess the siRNA loading capacity of PEI-HSNs, the amount of free siRNA left in the supernatant was determined, which decreased quickly with increasing weight ratio of PEI-HSNs to siRNA. The gel electrophoresis results further confirmed the ability of PEI-HSNs to encapsulate siRNA. The typical western blot analysis results showed that the expression of GAPDH proteins has been significantly inhibited by PEI-HSNs/siRNA (64%). Though a little lower, the transfection efficiency of PEI-HSNs was comparable to the commercial lipofectamine 2000. Furthermore, by adopting a vacuum-impregnation technique, superparamagnetic iron oxide nanoparticles (SPION) with a diameter of about 5 nm could be readily collected within the pores of HSNs, due to the large-sized pores of HSNs. The obtained SPION-HSNs exhibited superparamagnetic properties...
without magnetic hysteresis loops, which could be applied as contrast agents for $T_2$-weighted magnetic resonance imaging.

There are some special requirements in gene delivery in order to generate immune response. Subunit vaccines are much safer than traditional live attenuated and inactivated pathogens. However, these protein-based subunit vaccines are limited by the inefficient immune response without co-administration with an immune adjuvant, such as CpG oligodeoxynucleotides (CpG ODN). Recent studies have shown that the most potent therapeutic antigen-specific immune response can be generated only when antigens and CpG ODN were co-localized in the same antigen presenting cell (APC). Hence, Li et al. developed a novel europium-doped GdPO$_4$ hollow spheres/PEI core/shell nanoparticles for co-delivery of ovalbumin (OVA) and CpG ODN to the same APC, causing the activation of APCs along with effective antigen presentation and thus intensely enhanced the primary antigen-specific immune response.

To deliver OVA and CpG together to key cells and trigger the immune response, GdPO$_4$:Eu hollow spheres with an average pore diameter of 12.1 nm in the shell were prepared by etching the Gd(OH)CO$_3$:Eu colloidal spheres with NH$_4$H$_2$PO$_4$. After loading of OVA into the voids of hollow GdPO$_4$:Eu spheres, PEI was coated on the surfaces of GdPO$_4$:Eu spheres, forming a positively charged layer. CpG ODN was then attached on the surfaces of OVA-loaded GdPO$_4$:Eu spheres to contribute to the adsorption of DNA into the mesopores and the hollow cavity. The DNA bounded to nanoparticles was not cleaved by the endonucleases while free pEGFP-N2 was digested and cleaved into two chains by the enzymes under the same conditions, displaying that the hollow nanospheres could effectively protect DNA from degradation by nuclease. The lower in vitro cytotoxicity of GdPO$_4$:Eu HMNs was verified compared with a commercial gene transfection product lipofectamine 2000. Meanwhile, the transcriptional efficiency of the released plasmid DNA was comparable to that of Lipofectamine 2000. Moreover, the GdPO$_4$:Eu HMNs made it possible to trace cellular by both optical imaging and MRI. Therefore, the GdPO$_4$:Eu HMNs integrated with low cytotoxicity, stability, DNA protection and dual-mode imaging ability could serve as a new transmembrane carrier for the delivery of many biomolecules. It could be concluded that the GdPO$_4$:Eu HMNs could not only protect the plasmid DNA from degradation but also make it possible to track the location of the nanocarriers within the cells through fluorescence and MRI.

### 3.3 Biomedical imaging

Imaging has become an indispensable tool in the fields of medicine, diagnosis and therapy. Both the development of new...
modalities and the evolvement of the existing technologies have enabled the rapid diagnosis of disease through visualization and quantitative assessment. In many imaging modalities, contrast agents are needed to locate specific lesions or organs, to provide functional information, or to track the efficacy of a treatment. HMLS based contrast enhancement agents have demonstrated great potential in a variety of biomedical imaging such as optical imaging, magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US) imaging, and multimodal imaging.

3.3.1 Optical imaging. Optical imaging has been widely used for monitoring a variety of molecular/biological events in cells and tissues in superficial layers due to the low-cost, availability, operability and highly sensitive properties. Various strategies such as dye, quantum dots and upconversion nanoparticles doping or guest encapsulation have been developed to prepare HMLS for optical imaging in vitro and in vivo.

Shukla et al. prepared porous gold nanospheres via the galvanic replacement method with a dialysis bag to control the kinetics of reaction. As the reaction continued, the number of the pores per particle increased (Fig. 31) and the position of the pores still bore a correlation with the multiply twinned structure of the starting sacrificial Ag nanoparticles (Fig. 31A and C).

The Au HMLS have a significantly enhanced surface area compared with their solid counterparts and thus enhanced dye loading. By conjugation with the commonly used fluorescent dye, propidium iodide (PI), the porous Au nanospheres can be an excellent candidate for cell imaging. Even though PI is commonly used for imaging dead cells, it is interesting that the porous gold–PI conjugate could be uptaken by living cells as well in this study (Fig. 32). The same cell population was subsequently stained with Hoechst 33258 which is known to specifically work for only live cells. The significant overlap of the blue and red regions indicated that the PI loaded porous Au nanospheres indeed entered into living and viable cells. By contrast, no fluorescence was observed when live CHO cells were incubated with PI alone, indicating the inability of pure PI to enter into the living cells. It was speculated that the PI molecules in the inner regions of the pores in the Au HMLS would not be “seen” by the cells and entered the living cells by endocytosis along with the porous gold nanosphere carrier.

Considering the serious damage caused by organic dyes and ultraviolet (UV) excitation radiation, upconversion materials are more commonly used as contrast agents for optical imaging, which can effectively convert near-infrared wavelength excitation radiation into visible wavelength emission light with low bio-damages. Meanwhile, they possess other unique merits such as the low autofluorescence background, high penetration depth, and temporal resolution. Dong et al. synthesized Y2O3:Yb3+/Er3+ hollow nanospheres (UCHNS) with sizes less than 200 nm and pores (about 23 nm) in the shell. The PEG modified UCHNS (UCHNS@PEG) displayed good colloidal stability and biocompatibility. By tuning the molar ratio of the Yb/Er in the precursor, very pure red color could be obtained under excitation, which could penetrate deeper in tissue and had few adsorption by tissue chromophores. Fig. 33a–c show that the UCHNS were found in the cytoplasm confirming the feasibility of UCHNS for cellular imaging. In vivo test in mice showed that red color could be clearly observed under excitation at the injection positions by the naked eye (Fig. 33d and e). This novel nanomaterial has great potential in angiography due to the high contrast imaging and long blood circulation time.

However, the applicability of light-based imaging and therapies is confined to superficial tumors with shallow depths into the tissue due to the light penetration limitation. Thus, an emerging trend in the development of theranostic agents is the combination of optical imaging with other imaging modalities such as MRI, CT and ultrasound.

3.3.2 Magnetic resonance imaging. MRI is one of the most useful diagnostic modalities because it can noninvasively acquire three-dimensional tomographic information for whole tissue samples with high spatial resolution. Both T1- and T2-weighted MRI contrast agents have been developed to provide negative and positive contrast, respectively, such as

**Fig. 31** Representative high-magnification TEM images of Ag NPs inside the dialysis bag as a function of time of reaction with 5 × 10−6 M HAuCl₄ solution: (A) 0 h, (B) 3 h, and (C) 5 h. The arrows in the A point to the twin boundaries on the surface of silver nanoparticles, which are the preferable site of leaching of silver as a result of the replacement reaction, leading to the formation of cavities (arrows in B and C). Reprinted with permission from ref. 94. Copyright 2005 American Chemical Society.

**Fig. 32** Confocal microscopy images of live Chinese hamster ovary (CHO) cells stained with the porous gold–PI conjugate followed by Hoechst staining for checking cell viability. (A and B) Correspond to the phase contrast and fluorescence images respectively of CHO cells stained with the porous gold PI conjugate material. Subsequent staining with Hoechst gives blue fluorescence from live CHO cells (C) while superposition of images (B) and (C) is shown in (D). (E and F) Correspond to the phase contrast and fluorescent images respectively of live CHO cells stained with pure PI. Reprinted with permission from ref. 94. Copyright 2005 American Chemical Society.
iron oxide based nanomaterials and gadolinium oxide based nanomaterials.153–155

Xia’s group prepared PS hollow beads with holes on the surfaces by swelling commercial PS latex beads with toluene, followed by the quick freezing and drying strategy.64,65,148 The PS hollow beads were employed as containers for quick loading and encapsulation of different contrast enhancement agents due to the presence of large holes in the shell. Because the hole on the surface could be sealed by using a thermal annealing process (Fig. 34), the contrast agents were successfully encapsulated.

Fig. 35a shows the 19F MR spectrum of the centrifuge tube containing a suspension of perfluorooctane (PFO)-encapsulated PS hollow beads. The two major 19F peaks were attributed to the fluorine atoms in PFO and perfluoro-crown ether (PFCE, used as an internal standard), respectively. Furthermore, MR images in 1H (Fig. 35b) or 19F (Fig. 35c) mode from the same sample as Fig. 35a were obtained. It could be found that the suspension appeared bright, except for the small portion at the tip of the tube in 1H mode in Fig. 35b, while in 19F mode in Fig. 35c, a complementary image was observed, i.e. only the small portion at the tip of the tube was bright and the suspension was dark. Quantitative analysis demonstrated the high encapsulation efficiency of PFO in the PS hollow beads. Similarly, the saline-encapsulated and iodinated contrast compound-encapsulated PS hollow beads could be used as a contrast agent for thermo-acoustic tomography and micro-CT imaging, respectively.

3.3.3 CT imaging. As a noninvasive clinical diagnostic tool, X-ray CT possesses the ability of high spatial resolution and 3D visual reconstruction, which play a key role in disease diagnosis even with a high radiation dose. Previously, Park et al. synthesized HGNP with large-through-holes in the shell via galvanic replacement reaction for triple combination therapy and CT imaging.100 They compared the X-ray adsorption of DOX–HGNP...
and Ultravist 300, which is a widely used iodine-based CT contrast agent. As shown in Fig. 36A, X-ray absorption of DOX–HGNP increased linearly with increasing concentrations of DOX–HGNP. And 4.18 mg Au mL⁻¹ (21 mM) of DOX–HGNP had an equivalent Hounsfield unit (HU) value of 22.12 mg I mL⁻¹ (174 mM) of Ultravist 300 (Fig. 36B). As compared in Fig. 36C and D, it could be seen that injected DOX–HGNPs were as clearly visualized as Ultravist 300. Thus, DOX–HGNP could be a good candidate as a CT imaging contrast agent since no toxicities were observed with higher concentrations than 4 mg Au mL⁻¹, while 22 mg I mL⁻¹ of Ultravist 300 was the recommended dose for mice weighing 25 g. Moreover, the DOX–HGNP could be further modified with targeting agents to improve its imaging ability.

3.3.4 Ultrasound imaging. US imaging has been widely accepted for clinical diagnosis due to the features of non-invasiveness, excellent soft-tissue resolution, real-time imaging, high safety and convenience.156 Contrast agents have been employed in US imaging by providing enhanced backscattering from blood or tissue. Typically, the contrast agent is based upon microscale bubbles, which are effective scatterers of ultrasound waves.157 Novel kinds of US imaging contrast agents have been designed and fabricated to enhance the US imaging sensitivity.158–160

Bai et al. synthesized colloidal conducting PPy or PANi hollow spheres with a single large hole in the shell using commercially available PS beads via a shell-breaking method.157 Meanwhile, a pinhole was created in the surface during the dissolution of the PS beads. The synthesized hollow spheres have smooth, uniform shells and precisely controllable wall thickness, which make them particularly attractive to be used as contrast enhancement in ultrasonic imaging. As shown in Fig. 37, there were many bright, visible white dots located in the agarose template. It is worth noting that these bright dots were gathered and formed a crescent because the hollow spheres were push to move in the direction of ultrasound propagation when a 2 μm hollow sphere was used (Fig. 38a). However, when it came to 80 nm hollow spheres, a weaker contrast enhancement but more homogeneously distributed white dots were formed (Fig. 38b and c).

3.3.5 Multimodal imaging. With the intensive study and development of various multifunctional HMLS, other imaging modalities such as positron emission computed tomography (PET), thermoacoustic tomography (TAT), and photoacoustic (PA) imaging also have attracted the interest of researchers.157,161 Individual imaging modality (e.g. optical, MRI, CT and US) has its own advantages and drawbacks. However, when used in combination, they may allow for earlier detection of disease, better characterization of the condition, as well as better tracking of the effects of treatment.162 For example, PET/CT has demonstrated great advantages in finding the original position of the malignant tumor due to the high resolution of CT, which is difficult for PET alone to determine the exact location of lesions. Thus, HMLS with multimodal imaging features may play a key role in diagnosis and therapy.

Lim et al. fabricated multifunctional silica nanocapsules with a single hole in the shells which contained magnetic nanoparticles and NIR-emitting quantum dots (QDs) (Fig. 38).163 Even though the Fe₃O₄ and CdSe/ZnS QDs embedded in the shells of the silica nanocapsules were confirmed by elemental analysis, the presence of magnetic nanoparticles and QDs was further shown by illuminating magnetically separated samples with a UV light source (366 nm multiband), bright green emission was clearly observed. It is worth noting that strong fluorescence was generated from the shell region of the silica nanocapsules with the line intensity profile analysis, which further confirmed the locations of the QDs embedded.

Cellular and macroscopic fluorescence images of dendritic cells (DCs) labeled with silica nanocapsules containing Fe₃O₄ nanoparticles and CdSeTe/ZnS QDs could be seen in this study. After injecting the silica nanocapsules labeled DCs into the mice, the NIR fluorescence signal was obviously detected in the injection site. Furthermore, as shown in Fig. 39, MRI signals were also detected from the silica nanocapsules labeled DCs in this study. The observed signal intensity decreased owing to

![Fig. 36](image1)

**Fig. 36** In vitro and in vivo micro-CT images. (A) CT images of air, distilled water and DOX–HGNPs as a function of concentration. (B) X-ray absorption of DOX–HGNP and Ultravist 300. (C) Cross-sectional CT image in the back after injection of 4.18 mg Au mL⁻¹ of DOX–HGNP. (D) Cross-sectional CT image in the back after injection of 22.12 and 11.06 mg I mL⁻¹ of Ultravist 300. Reprinted with permission from ref. 100. Copyright 2015 Elsevier B.V.

![Fig. 37](image2)

**Fig. 37** Ultrasound images (in vitro test) with PANi hollow spheres of different sizes as the contrast agent: (A) the 2 μm PANi hollow spheres at a loading of 5.68 × 10⁷ particles per mL, (B) the 80 nm PANi hollow spheres at a loading of 2.27×10⁷ particles per mL, and (C) the 80 nm PANi hollow spheres at a loading of 2.27×10⁸ particles per mL. Reprinted with permission from ref. 157. Copyright 2009 Wiley-VCH.
the shortening of \( T_2 \). The darkened image confirmed the presence of DCs labeled with the silica nanocapsules in the injection site.

From the view of bioimaging application, upconversion phosphors emitted by NIR light have obvious advantages over organic dyes and quantum dots, which are less harmful to cells, minimize autofluorescence from biological tissues and penetrate tissues to a greater extent.\(^{164}\) Tian \textit{et al.} successfully prepared rare-earth doped gadolinium oxide (Gd\(_2\)O\(_3\)) hollow nanospheres with holes in the shell.\(^{165}\) These spheres emitted bright multicolored upconversion emissions under 980 nm laser excitation. And the optical imaging could be finely tuned from green to red by adjusting the codoped Yb/Er ratio. Moreover, these Gd\(_2\)O\(_3\) nanospheres brightened the \( T_1 \)-weighted images suggesting that Gd\(_2\)O\(_3\):Yb/Er hollow spheres could serve as a dual-imaging agent for optical/MR imaging. Because of the hollow cavity, these spheres could be used as drug delivery carriers. Thus, by combination of bimodal imaging and drug delivery capabilities, the as-prepared Gd\(_2\)O\(_3\) hollow spheres could be employed for diagnosis and therapy simultaneously.

### 3.4 HMLS-based theranostics

Theranostics refers to the fusion of therapy and diagnostics, with the purpose of optimizing efficacy and safety.\(^{131}\) Theranostic nanomedicine is an integrated nano-platform which can diagnose, deliver targeted therapy and monitor response to therapy.\(^{133}\) Many multifunctional HMLSs, capable of performing one or more of the above duties, have attracted great interest for theranostic purpose.

Deng \textit{et al.} fabricated Yb\(^{3+}\)/Er\(^{3+}\)/Mn\(^{2+}\) co-doped hollow CaF\(_2\) nanospheres (UCHNs, Fig. 40) by a hydrothermal route.\(^{166,167}\) Then the hollow cavities were filled with 2-aminoethyl methacrylate hydrochloride (AMA) monomers, which formed PAMA by photo-initiated polymerization. The fabricated UCHN–PAMA hybrid microspheres provided the up-converting luminescence (UCL) imaging modality. Meanwhile, the presence of functional Mn\(^{2+}\) and Yb\(^{3+}\) offered the enhanced \( T_1 \)-weighted MR and computed tomography (CT) imaging, respectively.

Owing to the active amine groups on PAMA, the carboxylic functionalized Pt(\(\text{IV}\)) pro-drug could be decorated to PAMA in the hollow cavities, forming the UCHN–PAMA–Pt(\(\text{IV}\)) nanodrug. \textit{In vitro} cell cytotoxicity tests revealed that UCHN–PAMA–Pt(\(\text{IV}\)) had the strongest inhibition ability to Hela cells and a better suppression effect on cancer cells than small molecules of cisplatin and the Pt(\(\text{IV}\)) pro-drug at low drug concentration. \textit{In vivo} anti-tumor tests on small mice demonstrated that the UCHN–PAMA–Pt(\(\text{IV}\)) nanodrug was obviously superior to the control groups with respect to tumor suppression (Fig. 41) and the reducing side effect to normal tissue. UCHN–PAMA used as a drug carrier showed good biocompatibility for no apparent damages or changes in the detected organs between the UCHN–PAMA–Pt(\(\text{IV}\)) group and the control group \textit{in vivo}. Therefore, the UCHN–PAMA–Pt(\(\text{IV}\)) hybrid microspheres constructed a multifunctional platform for cancer therapy and diagnosis.

In their another work, highly uniform and well-dispersed CaF\(_2\) hollow spheres (300–930 nm) with mesopores (av 24.6 nm) in the shell were synthesized by Ostwald ripening.\(^{168}\) By a

---

**Fig. 38** Magnetic and optical properties of silica nanocapsules containing Fe\(_3\)O\(_4\) nanoparticles and CdSe/ZnS QDs. (a) Colloidal solution. (b) Image of the solution after magnetic separation. (c) Green fluorescence image of the sample shown in (b). (d) Confocal fluorescence image of the sample. Line intensity profile analysis showed that strong fluorescence was generated from the shell region of the silica nanocapsules. (e) SEM image, and (f) TEM images of silica nanocapsules. Reprinted with permission from ref. 163. Copyright 2009 Wiley-VCH.

**Fig. 39** (a) Prussian-blue-stained images of DCs labeled with the silica nanocapsules containing Fe\(_3\)O\(_4\) nanoparticles and CdSeTe/ZnS QDs. (b) MRI image of the sample from (a). (c) MRI image of a mouse injected with labeled DCs via a subcutaneous route. Reprinted with permission from ref. 163. Copyright 2009 Wiley-VCH.
similar strategy, Ce³⁺/Tb³⁺-codoped CaF₂ hollow spheres were also prepared, which showed strong green photoluminescence. When the hollow CaF₂:Ce³⁺/Tb³⁺ spheres used as drug carriers, the emission intensity of Tb³⁺ in the drug carrier system varied with the released amount of ibuprofen, which could easily track and monitor the drug release according to the change in luminescence intensity.

Zheng et al. fabricated polyethyleneimine (PEI) functionalized YbPO₄:Er upconversion porous nanospheres (UCPSs) with a pore of ca. 22 nm. The capabilities of upconversion luminescence (UCL)/CT bimodal imaging were attributed to the transitions of Er³⁺ ions and the high atomic number and strong X-ray attenuation of Yb³⁺ ions, respectively. It could be concluded that PEI–UCPSs provided higher CT contrast than a commercial iodine-based contrast agent (iopromide) due to the Yb³⁺ ions being more efficient in X-ray absorption than iopromide. It demonstrated that the CT contrast was clearly enhanced after injection with different PEI–UCPSs. Moreover, these PEI–UCPSs showed low cytotoxicity, good anti-cancer properties and high drug loading capacity, indicating their great potential in cancer theranostic.

Combination therapy can also be realized by using multifunctional HMLS, which has shown a significantly greater therapeutic effect than monotherapy. Utilizing the unique optical and morphological properties of gold HMLS, Park et al. synthesized DOX–HGNPs for the triple combination therapy (chemotherapy, thermal and radiotherapy) and CT imaging, simultaneously. This triple combination therapy dramatically delayed tumor growth by 4.3-fold and reduced tumor’s weight by 6.8-fold compared to control tumors. Moreover, they demonstrated the feasibility of DOX–HGNPs as a CT contrast agent. Thus, it was possible that the CT-guided thermal or radiation therapy might be realized based on this multifunctional DOX–HGNPs.

4. Conclusions and outlook

This review highlighted and discussed the syntheses and biomedical applications of HMLS. Various synthetic methods were classified into the template-directed approach, Ostwald ripening, shell-breaking method and galvanic replacement reaction, according to the formation mechanism of the large-through-hole in the shell. The as-synthesized HMLS have showed broad prospects in the field of biomedical applications including adsorption and encapsulation of proteins, drug/gene delivery, biomedical imaging and theranostics attributed to their hollow cavity and large-through-holes in the shell. Moreover, multifunctional hollow porous microspheres can also be obtained through engineering the preparation process by regulating the compositions, structure, and surface properties. These results are attractive and encouraging since better results and breakthroughs are emerging successively.

However, tremendous efforts are further needed prior to getting a “perfect” functional material. First, it can be seen that some harmful solvents and chemicals were still used in the synthesis process of HMLS. To utilize in clinical applications, materials should be low toxic or approved by medical administrations. Silica, gold, iron oxide, PLA and polysaccharides are considered suitable candidates because they are biocompatible and low toxic. Furthermore, some biomass existing in humans and animals, like BSA, HSA and lysozyme, may be excellent alternatives to harmful chemicals. The choice of raw materials to prepare HMLS is important and has an effect on the choice of the corresponding solvents and
other chemicals to use once the composition of HMLS is fixed. It should be noted that toxic solvents, surfactants and other harmful chemicals, if cannot be avoided during preparation, should be easily removed from HMLS prior to in vivo applications. Otherwise, their chance for clinical application is little since the toxicity can be resulted from both the naked material and the residual toxic substances within them. The in vivo biocompatibility, toxicity, clearance of HMLS and long-term effects also need to be further evaluated.\(^{172}\) Besides, the morphology and surface properties of HMLS can also affect their biomedical applications.\(^{177-179}\) For the morphology of HMLS, the diameters of both the microspheres and the internal cavity, the sizes and numbers of holes and their distribution in the shell are expected to be controlled precisely. The shell-breaking method is mainly suitable for the preparation of polymer HMLS. In some cases, the organic materials used in this method must be capable of swelling or shrinkage. The limitation of Ostwald ripening lies in the less controllable morphology of HMLS. And the diameters of HMLS are quite inhomogeneous, which also limits its biomedical applications with special demands. While the galvanic replacement reaction is confined to preparation of HMLS composed of noble metal, transition metal oxides or sulfides, and the residual toxicant elements in the HMLS may cause severe damage to cells. Accordingly, more, and intelligent, versatile and bio-friendly methods for the synthesis of HMLS with controllable compositions and morphologies are highly desired. In particular, we would believe that the following several aspects, but not limited, should be paid more attention in the future research and application of HMLS: (i) hybrid materials when engineering a product to meet all the holistic performance requirements of HMLS should be more focused; (ii) to achieve controlled release of functional guest materials at the destination site, targeting has been playing a key role in diagnosis and therapy. Surface modifications of HMLS may be an efficient way to obtain enhanced circulation and targeting effects in vivo; (iii) even though larger holes are preferable in some biomedical applications, they may bring the leakage problem in some cases. Hence, precisely controlling the morphology of HMLS is still challenging. We hope that the general synthetic principles and bio-applications of HMLS, as well as the selected references highlighted in this review, and our personal opinions will provide the readers with the necessary background and ideas to develop deeper expertise and more effective fabrication methods for both syntheses and applications of HMLS. Though there are many difficulties, if finely engineered, better performances and more applications of HMLS are believed to be achieved by this multifunctional platform route in the near future.

Acknowledgements

Financial support of this research from the National Natural Science Foundation of China (51322307, 51273218, 51133001 and 21374018), National "863" Foundation (2013AA031801), Science and Technology Foundation of Ministry of Education of China (20110071130002), Science and Technology Foundation of Shanghai (13JC1407800) and "Shu Guang" project supported by Shanghai Municipal Education Commission and Shanghai Education Development Foundation are appreciated.

Notes and references


95 A. M. Goodman, Y. Cao, C. Urban, O. Neumann, C. Ayala-Orozco, M. W. Knight, A. Joshi, P. Nordlander and N. J. Halas, ACS Nano, 2014, 8, 3222.


113 V. P. Chauhan and R. K. Jain, Nat. Mater., 2013, 12, 958.


127 F. Zhao, Y. Zhao, Y. Liu, X. Chang, C. Chen and Y. Zhao, Small, 2011, 7, 1322.


129 A. Verma and F. Stellacci, Small, 2010, 6, 12.


