Journal of Controlled Release 172 (2013) 1075–1091

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Gelatin-based nanoparticles as drug and gene delivery systems: Reviewing three decades of research

Ahmed O. Elzoghby *

Department of Industrial Pharmacy, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt

ARTICLE INFO

Article history:
Received 26 July 2013
Accepted 21 September 2013
Available online 2 October 2013

Keywords:
Gelatin nanoparticles
Drug delivery
Gene delivery
Pharmaceutical applications
Surface-modifications
Gelatin nanocomplexes

ABSTRACT

Gelatin is one of the most versatile natural biopolymers widely used in pharmaceutical industries due to its biocompatibility, biodegradability, low cost and numerous available active groups for attaching targeting molecules. These advantages led to its application in the synthesis of nanoparticles for drug and gene delivery during the last thirty years. The current article entails a general review of the different preparation techniques of gelatin nanoparticles (GNPs): desolvation, coacervation-phase separation, emulsification-solvent evaporation, reverse phase microemulsion, nanoprecipitation, self-assembly and layer-by-layer coating, from the point of view of the methodological and mechanistic aspects involved. Various crosslinkers used to improve the physicochemical properties of GNPs includintg aldehydes, genipin, carbodiimide/N-hydroxysuccinimide, and transglutaminase are reported. An analysis is given of the physicochemical behavior of GNPs including drug loading, release, particle size, zeta-potential, cytotoxicity, cellular uptake and stability. This review also attempts to provide an overview of the major applications of GNPs in drug delivery and gene therapy and their *in vivo* pharmacological performances, as well as site-specific drug targeting using various ligands modifying the surface of GNPs. Finally, nanocomplexes of gelatin with polymers, lipids or inorganic materials are also discussed.

Contents

* Corresponding author. Tel.: +20 3 3825212; fax: +20 3 4873 273.
E-mail address: dr_ahmedelzoghby@yahoo.com.

0168-3659/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jconrel.2013.09.019

1. Introduction

Nanoparticles made of biodegradable polymers like proteins and polysaccharides can act as efficient drug delivery vehicles for controlled and targeted release, aiming to improve the therapeutic effects and also to reduce the side effects of the formulated drugs [1]. Over the past few decades, there has been considerable interest in developing protein-based nanoparticles as GRAS (generally regarded as safe) drug delivery devices. The underlying rationale is their exceptional characteristics, namely biodegradability, nonantigenicity, high nutritional value, abundant renewable sources, extraordinary binding capacity of various drugs and possibility of less opsonization by the reticuloendothelial system (RES) through an aqueous steric barrier in addition to greater stability during storage and *in vivo* [2].

Gelatin is a denatured protein that is obtained either by partial acid or alkaline hydrolysis of animal collagen. Having a long history of safe use in pharmaceuticals, cosmetics, as well as food products, it is considered as GRAS material by the United States Food and Drug Administration (FDA) [2,3]. For systemic administration, gelatin has a great deal of experience for its use in parenteral formulations. It is used clinically as a plasma expander and included as a stabilizer in a number of protein formulations, vaccines and gelatin sponge (Gelfoam®). Gelatin offers the advantages of being cheap and readily available. It has attracted a great interest for its biocompatibility and biodegradability. Moreover, gelatin has relatively low antigenicity because of being denatured in contrast to collagen which is known to have antigenicity due to its animal origin [2,3]. Moreover, gelatin does not produce harmful byproducts upon enzymatic degradation, as it is derived from collagen, which is the most abundant protein in animals. Finally, gelatin chains contain motifs such as Arg-Gly-Asp (RGD) sequences that modulate cell adhesion, thereby improving the final biological behavior over polymers that lack these cell-recognition sites [4]. Due to their intrinsic protein structure with the high number of different accessible functional groups, they bear multiple modification opportunities for coupling with crosslinkers and targeting-ligands which may be especially useful in developing targeted drug delivery vehicles. In addition, gelatin as a matrix for mineralization has evoked a lot of interest in the field of tissue engineering [4,5].

Overall, its biodegradability, biocompatibility, chemical modification potential and cross-linking possibility make gelatin-based nanoparticles (GNPs) a promising carrier system for drug delivery. This review paper will focus on GNPs used as vectors for drug and gene delivery. The structure of gelatin, preparation and characterization techniques of GNPs, drug delivery applications, and their surface modification with various ligands are discussed. Further, gelatin nanocomplexes with other polymers, lipids or inorganic materials are elaborated.

2. Chemical structure

Gelatin is a polyampholyte having both cationic and anionic along with hydrophobic groups present in the approximate ratio 1:1:1, which makes this polypeptide special. The gelatin molecule is ~13% positively charged (lysine and arginine), ~12% negatively charged (glutamic and aspartic acid) and ~11% of the chain hydrophobic in nature (comprising leucine, isoleucine, methionine and valine). Glycine, proline and hydroxyproline form the rest of the chain. The representation $(Gly\text{-}X\text{-}Pro)_n$ is responsible for the triple helical structure of gelatin, where X represents the amino acid, mostly lysine, arginine, methionine and valine ~6%. One third of the chain is comprised of glycine ~33% and another one third is either proline or hydroxyproline ~33%. The rest are other residues. Commercially, gelatin is available as both cationic (gelatin type A, isoelectric point (pI) 7–9, prepared by an acid hydrolysis of pig skin type I collagen) or anionic (gelatin type B, pI 4.8–5, prepared by an alkaline hydrolysis of bovine collagen) protein without the necessity of additional functionalization [3,6]. Fig. 1 depicts the composition of gelatin in terms of amino acids.

3. Preparation of GNPs

GNPs have been richly documented in the literature as carrier system for drug and gene delivery. Since first described in 1978 [7], various methods have been used to prepare GNPs.

3.1. Desolvation

Desolvation technique is based on the addition of a desolvating agent (*e.g.*, alcohol or acetone) to an aqueous gelatin solution in order to dehydrate the gelatin molecules resulting in conformational change from stretched to coil conformation. Next, to harden the native particles, a step of crosslinking is required [3,8]. However, the use of native gelatin produces large particles with a wide size range due to heterogeneity in molecular weight of gelatin. Addition of a second desolvation step by Coester et al. [9], has been shown to be more efficient in the formation of smaller and uniform nanoparticles. The high molecular weight (HMW) gelatin was precipitated in the first desolvation step to remove the low molecular weight (LMW) gelatin then HMW gelatin is re-

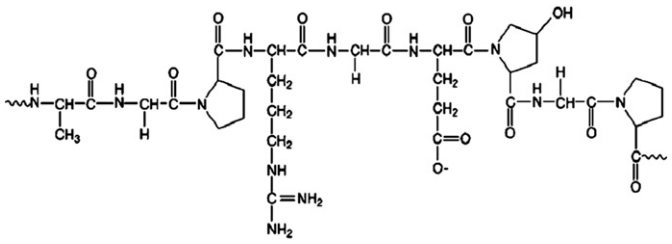

Fig. 1. Basic chemical structure of gelatin [3].

dissolved and desolvated again. A new simplified one-step desolvation approach was developed by Ofokansi et al. [10], in which it is no longer necessary to perform an initial desolvation step to discard the LMW gelatin fraction. In this method, before desolvation, the pH of gelatin solution was adjusted to neutral values of 7.0 clearly above the pI so that gelatin molecules would be sufficiently uncharged to remain sensitive to desolvation but sufficiently charged to prevent their aggregation. Moreover, a preparation temperature of 37 °C was also selected to ensure that the molecular weight distribution of gelatin remained relatively constant during incubation [10]. GNPs with a size of 253–479 nm and polydispersity index (PDI) of 0.073 were obtained. Although extensively used to prepare GNPs, desolvation technique has two major drawbacks: the use of organic solvents and the use of toxic crosslinkers.

3.2. Coacervation-phase separation

Coacervation is a process during which a homogeneous solution of charged macromolecules undergoes liquid–liquid phase separation, giving rise to a polymer rich dense phase at the bottom and a transparent solution above [6]. The addition of natural salt or alcohol normally promotes coacervation that resulted in desired nanoparticles. GNPs (600 to 1000 nm) were successfully prepared by slow addition of sodium sulfate to aqueous gelatin solution containing surfactant (Tween 20) followed by addition of isopropanol to dissolve the precipitate by sodium sulfate [11]. A second aliquot of sodium sulfate was added until the solution turned turbid, which indicated the formation of gelatin aggregates. Distilled water was then added until the solution turned clear and glutaraldehyde (GA) was added to crosslink GNPs.

3.3. Emulsification-solvent evaporation

In this technique, GNPs (100 to 400 nm) were prepared adopting a solvent evaporation method based on a single W/O emulsion. Aqueous phase containing both gelatin and drug was mixed with vigorous shaking with the oil phase *e.g.* organic solution of polymethylmethacrylate [12–14] or paraffin oil [15] followed by crosslinking with GA [12–14] or genipin [15]. A novel water-in-water emulsion technique was recently used to prepare insulin-loaded GNPs (250 nm) under mild conditions which could guarantee the bioactivity of insulin [16]. Briefly, a prewarmed gelatin solution containing insulin was added dropwise to poloxamer solution under stirring to form an emulsion which was then cooled to 5 °C to promote nanoparticle formation followed by crosslinking.

3.4. Reverse phase microemulsion

In this method, aqueous gelatin solution was added to solution of the surfactant, sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in n-hexane, then GA was added to crosslink the nanoparticles followed by evaporation of n-hexane for recovery of GNPs [17]. The surfactant AOT when dissolved in non-polar solvents like hexane forms reverse micelles where hydrophobic tails of surfactants are assembled towards the bulk non-polar solvent and hydrophilic head is directed away from the bulk solvent inside enclosing an aqueous core in which the aqueous solution of gelatin and crosslinker was dissolved and so the GNP formation and crosslinking take place inside the inner aqueous core of reverse micellar droplets. The size of the inner aqueous core of reverse micelles is in nanometer range so the GNPs prepared inside these nanoreactors have average diameter of 37 nm. The advantage of utilizing this type of microemulsion system for nanoparticle formation is that the size of nanoparticles can be controlled by modulating the size of aqueous micellar core [18]. The entrapment efficiency of the nanoparticles for fluorescein isothiocyanate-dextran as a fluorescent marker was found to be approximately 90%.

3.5. Nanoprecipitation

In nanoprecipitation technique, water as solvent phase (containing gelatin and drug) was added slowly to ethanol as the nonsolvent phase containing poloxamer as a stabilizer then GA was added to crosslink the nanoparticles [19,20]. The nanoparticles turned out narrowly distributed around unimodal size mean of 251 nm with unimodal polydispersity of 0.096. The mechanism of formation of nanoparticles has been explained by the interfacial turbulence generated during solvent displacement. Subsequently a violent spreading is observed because of mutual miscibility between the solvents. Droplets of solvent, probably of nanometric size, are torn from the interface. These droplets are rapidly stabilized by the stabilizing agent, until diffusion of the solvent is complete and protein solidification has occurred [19,20]. Nanoprecipitation presents numerous advantages, in that it is a straightforward technique, rapid and easy to perform. It often enables the production of small nanoparticles with narrow unimodal distribution. Moreover, it does not require extended shearing rates, sonication or very high temperatures, and is characterized by the absence of oily–aqueous interfaces [21,22].

3.6. Self-assembly

Nanoparticles can be formed through self-assembly of gelatin molecules through one of the following methods:

i. *Chemical modification.* The structure of hydrophilic gelatin makes it possible to chemically conjugate with various hydrophobic molecules to form an amphiphilic polymer. The hydrophobically modified gelatin is capable of undergoing conformational rearrangement upon dissolving in an aqueous environment thus self-assembling to form micelle-like nanospheres where the hydrophobic segments would aggregate inward forming a hydrophobic core entrapping hydrophobic therapeutic molecules with a hydrophilic outer shell [23]. Self-assembled GNPs were first proposed by Kim and Byun [24], where the carboxyl groups of deoxycholic acid (DOCA) and carboxylated monomethoxy polyethylene glycol (MPEG) were coupled with amine group of gelatin by dichlorohexylcarbodiimide (DCC) method. The synthesized gelatin/DOCA/MPEG conjugates were ultrasonicated

to produce self-assembled nanoparticles where DOCA acted as the hydrophobic core, thereby aggregating gelatin molecules and hydrophilic MPEG chains located at the surface of the nanoparticles. Hexanoyl anhydride and alpha-tocopheryl succinate (TOS) were utilized as hydrophobic groups to chemically modify the hydrophilic gelatin [25], (Fig. 2) or recombinant human gelatin (rHG) [26], respectively [25,26]. For loading of lipophilic drug into the hydrophobic core of the nanoparticles, camptothecin [25] or 17-AAG (17-allylamino-17-demethoxygeldanamycin) [26] was mixed with the amphiphilic copolymer solution under sonication. Free drug was removed by centrifugation or dialysis followed by lyophilization. A very low CAC (0.00216 mg/mL) was observed for hexanoyl-modified gelatin in aqueous solutions which ensured structural stability of the nanoparticles in blood circulation [25]. According to Tanigo et al. [27], simvastatin was water solubilized by incorporation into L-lactic acid oligomer (LAo)-grafted gelatin micelles. The micelles were then mixed with gelatin, followed by chemical crosslinking of gelatin to obtain gelatin hydrogels. In the presence of collagenase, the hydrogels are degraded enzymatically to make gelatin water soluble, resulting in the sustained release of simvastatin. In another study, the water soluble drug, doxorubicin hydrochloride (DOX) could be incorporated into amphiphilic gelatin-co-poly(lactide)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer nanoparticles *via* double emulsion or nanoprecipitation method [28].

ii. *Simple mixing.* Gelatin and drug solutions were directly mixed to allow their interaction based on specific forces without chemical modification of gelatin. Hydrogen bonding was mainly responsible for self-assembly of nanoparticles of gelatin with tea catechins [29] or partially purified ellagitannins (PPE) [30] upon simple mixing. Hydrophobic interactions are also suggested to be contributing to the self-assembly of nanoparticles. It was known that the protein with compact tertiary structure provides less hydrophobic sites, constraining interaction with tannin molecules. However, gelatin is a proline-rich protein with extended random coil conformation. Hence, gelatin provides more interaction sites for tannin molecules, eventually promoting higher affinity for tannin molecules [30].

3.7. Layer-by-layer (LbL) coating

In this technique, GNPs (200 nm) were coated by alternating layers of oppositely charged anionic and cationic polyelectrolytes (polystyrene sulfonate/polyallylamine hydrochloride, polyglutamic acid/poly-L-lysine, dextran sulfate/protamine sulfate, carboxymethyl cellulose/gelatin, type A). Surface charge alternation with sequential deposition of polycation and polyanion layers was observed [31,32]. Different polyphenols were loaded into the GNPs by adsorption from their concentrated solutions. Adsorption of polyphenols with higher molecular weights and a larger number of phenolic groups was found to be higher (70% for theaflavin). Modification of nanoparticle surfaces with polyelectrolyte LbL shells allows for modulating nanoparticle cell uptake, providing a template for their modification with tumor-targeting agents, increasing colloidal stability, and controlling loading/release characteristics [31].

4. Crosslinking of GNPs

Crosslinking of GNPs is required to give gelatin stability, shape and an enhanced circulation time *in vivo* as compared to uncrosslinked particles [2,3]. GNPs prepared without crosslinking were found to be unstable and tended to aggregate upon aging.

4.1. Aldehydes

As reported previously [33], using *glyoxal* to crosslink GNPs resulted in instantaneous mass aggregation and precipitation of the nanoparticles. Hence, number of investigations showed *glutaraldehyde* (GA) as an effective crosslinker for GNPs [10,34]. GA crosslinking did not induce aggregation of GNPs which remained stable for more than 10 months on storage at 2–8 °C. GA is a non-zero length crosslinker which induces poly- or bi-functional crosslinks into the network structure of proteins by bridging free amino groups of lysine or hydroxylysine residues [34]. Because GA was consumed during GNP crosslinking and residuals were removed by particle purification, no adverse effects could be observed. Thus, GA-crosslinked GNPs did not trigger undesired immune or toxicological reactions. However, a reduction in biocompatibility of crosslinked gelatin films was reported earlier. Slight toxicity was demonstrated in oral toxicology studies as well as contact dermatitis if applied on the skin was shown. Therefore, the use of non-toxic crosslinking agents seems important for future applications of GNPs [35,36].

D,L-Glyceraldehyde can be considered a non-toxic crosslinking agent. The D-form of this aldehyde is phosphorylated by triokinase in the human body forming D-glyceraldehyde-3-phosphate that enters in glycolysis cycle. In the last years, gelatin crosslinked with D,L-glyceraldehyde was proposed as a new material for pharmaceutical applications [37]. In the study of Zhao et al. [16], insulin-loaded D,L-glyceraldehyde-crosslinked GNPs were prepared for pulmonary administration. No toxicity was reported after subcutaneous or intratracheal administration of nanoparticles into rats.

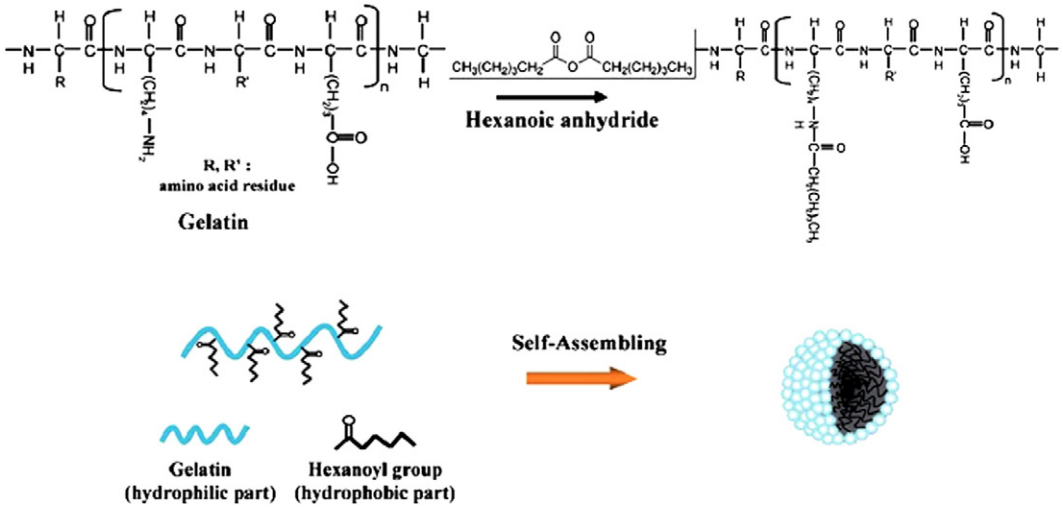

Fig. 2. The reaction scheme for the synthesis of self-assembled hexanoyl-modified GNPs [25].

4.2. Genipin

Genipin is a natural crosslinker extracted from gardenia fruit that has been used in herbal medicine and fabrication of food dyes. Genipin requires a longer crosslinking time; however, its cytotoxicity is approximately 10,000 times less than GA [38,39]. The mechanism of crosslinking reaction of proteins by genipin was proposed by Song et al. [40], to involve two free amino groups of lysine residue on the protein macromolecular chains crosslinking with one molecule of genipin. Genipin-crosslinked rHG nanoparticles were efficiently internalized in the cell without significant cytotoxicity [41]. Genipin was also used to fix the structure of gelatin–dextran micelles encapsulating tea polyphenol to avoid disintegration after dilution. The crosslinked micelles were stable with no considerable size change was found by 100-fold dilution [42].

4.3. Carbodiimide/N-hydroxysuccinimide (CDI/NHS)

A mixture of water soluble *carbodiimide and N-hydroxysuccinimide (CDI/NHS)* was successfully used as a non-toxic crosslinking system of GNPs [43]. The nanoparticles were smoother and more homogeneous with smaller size and narrower size distribution than those crosslinked by GA. Using paracetamol as a model drug, both drug entrapment and loading efficiencies were higher in the CDI/NHS crosslinked nanoparticles; however, the release kinetics was comparable to that of GA-crosslinked nanoparticles. The differences in the characteristics of CDI/NHS and GA-crosslinked GNPs were attributed to the different nature of network structures formed by the two crosslinking agents [43].

4.4. Microbial transglutaminase (MTG)

Enzymatic crosslinking is an attractive approach due to high specificity of the enzyme catalysis controllable to a certain degree by changing pH and temperature [44,45]. Recombinant microbial *transglutaminase* (MTG) was investigated by Fuchs et al. [46], to crosslink GNPs prepared by a two-step desolvation technique using acetone as a desolvating agent. This acyltransferase forms intra- and intermolecular isopeptide bonds in and between proteins by crosslinking the ε-amino groups of lysine to the side chain amide group of glutamine, thereby releasing one molecule of ammonia per crosslink. As TG is sensitive to organic solvents, purification of the acetone-containing particle dispersion was considered crucial after the second desolvation step prior to addition of the crosslinking enzyme. Crosslinking reactions were best at 25 °C using an ion-free solvent at a neutral pH and have been terminated after 12 h. Particles of defined size below 250 nm and narrow size distribution stable in a short-range stability set-up were produced successfully [46].

4.5. Other crosslinkers

Double-crosslinked gelatin–chitosan nanoparticles were prepared where part of the amino functional groups of both polymers was ionically-crosslinked with sodium sulfate for polymers gelation, forming an interpenetrated network [47]. The particles were further hardened by covalent crosslinking the free amino groups with GA. The use of an ionic crosslinker allows a significant reduction in the amount of covalent crosslinker, often toxic, but usually indispensable due to the required gel stability (Fig. 3). Narayanan et al. [48] used $CaCl_2$ for crosslinking GNPs. At a pH of 8.5, the –COOH groups of gelatin become deprotonated facilitating crosslinking with Ca^{2+} in $CaCl_2$. Concentration of $CaCl_2$ used was found to affect the particle size and drug entrapment.

5. *In vitro* characteristics of GNPs

5.1. Drug loading

Drugs or genes encapsulated into GNPs may be either entrapped into the matrix of nanoparticles during preparation or adsorbed onto the surface of the preformed nanoparticles. Hydrophilic drugs can be successfully loaded into GNPs by incubating the drug with aqueous gelatin solution for sufficient time prior to nanoparticle formation to allow drug–protein binding. Various mechanisms may be involved in drug loading into GNPs including physical entrapment, electrostatic attraction or covalent conjugation [3]. Furthermore, hydrogen bonding and hydrophobic interactions between drug and gelatin were also reported [29,30]. DXR could be incorporated into magnetic GNPs through electrostatic interactions between positively charged DXR and the negatively charged FeO^- and COO^- coating layers after allowing GNPs to swell in a freshly prepared drug solution [15]. Moreover, a covalent binding of DXR to the protein matrix *via* GA crosslinking was hypothesized. DXR-loaded GNPs showed a greater number of free amino groups than the unloaded ones mainly due to competition between the amino group of DXR and the amino groups of the gelatin chains during the crosslinking process [34]. In another study, the competition between carboxylic groups of gatifloxacin and aldehyde groups of GA to react with the amino groups of gelatin molecules resulted in reduction of gatifloxacin-loading efficiency into crosslinked GNPs compared to uncrosslinked ones [20].

For loading of hydrophobic drugs, a concentrated solution of the drug (*e.g.* amphotericin B [49] or hypocrellin B [50]) in water-miscible organic solvent (*e.g.* ethanol or dimethylsulfoxide) was added to gelatin solution under stirring and sonication for simultaneous desolvation and loading before crosslinking. Entrapment of hydrophobic drugs in GNPs could be explained on the basis of preferential localization of drug inside the nanoparticulate core, which was less hydrophilic than the outer aqueous environment [49]. In another study, the hydrophobic drug, hydrocortisone, was complexed with cyclodextrins in order to increase its aqueous solubility before mixing with gelatin solution [51]. Moreover, lipophilic drugs could be successfully loaded into the hydrophobic core of self-assembled amphiphilic gelatin nanocarriers with high loading [25,26]. The extent of drug loading and encapsulation efficiency of GNPs depends on the molecular weight and also on the nature of the substance incorporated. Studies by Truong-Le et al. [52] have shown that the encapsulation efficiency of GNPs increases with increasing molecular weight. Similarly, Saxena et al. [53] reported that encapsulation efficiency of cycloheximide was found to be 26, 34.1 and 41% in 75, 175 and 300 Bloom gelatin (molecular weights 22, 40 and 87.5 kDa) respectively.

5.2. Drug release

The drug release from GNPs may be due to three predominant mechanisms including desorption, diffusion and biodegradation of GNPs [3,54]. Many factors were shown to influence the rate of drug release from GNPs. The crosslinking density of gelatin was reported to have a significant influence on the drug release rate from nanoparticulate matrix. Bajpai and Choubey [14] found that both the fractional release of cytarabine and the swelling ratio increase with increasing GA concentration up to 10.6 mM while beyond it a fall in release and swelling was noticed. Since GA is a hydrophilic crosslinker, its increasing number of linkages in the nanoparticles enhances their hydrophilicity, which, will allow increasing number of water molecules to penetrate into the nanoparticle and obviously the swelling ratio and the release of cytarabine will increase. However, beyond 10.6 mM of GA, the size of nanoparticle will decrease due to enhanced crosslinking density, and therefore, both swelling and release will fall.

Another factor is the presence of proteolytic enzymes which accelerates the biodegradation of GNPs resulting in faster drug release. Leo

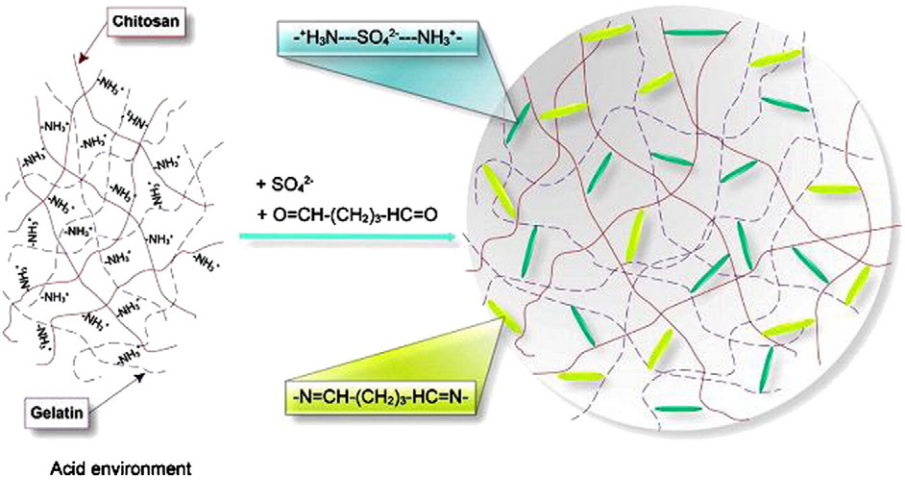

Fig. 3. Chitosan and gelatin particle co-crosslinking with sodium sulfate and GA [47].

et al. [55] have studied the release of DOX from GNPs by dynamic dialysis in both absence and presence of trypsin. Only a little fraction (from 9 to 13%) of the drug was released in the absence of the enzyme corresponding to the free drug fraction (Fig. 4). Addition of trypsin caused the release of a further 10–15% of the drug loading probably due to a fraction of the DOX–peptide conjugates produced by nanoparticle digestion and characterized by a molecular weight lower than membrane cut-off (3500 Da). The major part of DOX (about 70%) was bound to the protein matrix *via* GA, forming a drug-conjugate having a molecular weight higher than membrane cut-off so cannot diffuse freely through it.

5.3. Particle size and surface charge

Most GNPs prepared by the above methods have reported mean sizes ranging from 200 to 400 nm. Colloidal stability, drug encapsulation efficiency, loading capacity, drug release and biodistribution profile, cell internalization kinetics *etc.* are strongly influenced by the particle size. The effect of various parameters such as temperature, pH, degree of crosslinking, nature of gelatin and type of desolvating agent on the size of the GNPs has been investigated by several groups. Nahar et al. [49] showed that increasing the amount of GA caused a significant reduction in particle size of amphotericin B-loaded GNPs which could be attributed to crosslinking of free amine groups at the nanoparticle surface by GA, which caused hardening of particles leading to reduction in size. As the amount of crosslinker was increased, more groups were crosslinked and subsequently caused a higher degree of reticulation [34].

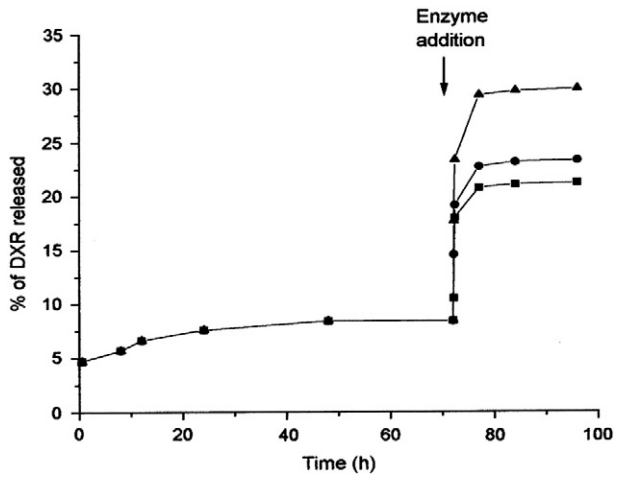

Fig. 4. Doxorubicin *in vitro* release from GNPs before and after treatment with different proteolytic enzymes; protease (▲); α-chymotrypsin (●); trypsin (■) [55].

A temperature of 40 °C was found to be the minimum as well as the optimum temperature resulting in GNPs with nanometric size and narrow size distribution because of the high viscosity of gelatin at room temperature. The possible reason for this effect is the triple-helical structure of gelatin, which uncoiled as temperature rose in a controlled manner. However, at higher temperatures (50 and 60 °C), an unexpected increase in particle size was observed, probably due to complete uncoiling of gelatin chains [49]. Additionally, formulation pH at the second desolvation step was significant to obtain GNPs of desired size and low PDI. pH 3 for type A or pH 11 for type B gelatin, was found to be optimum, because formation of GNPs probably was associated with a higher degree of electrostatic interactions causing charge neutralization and consequently formation of GNPs on adding desolvating agent to gelatin solution [53]. Such pH dependent behavior of gelatin could be attributed to its polyelectrolyte nature (contains both amino and carboxylate-terminated chains at its pI), and as the pH was shifted to the acidic or basic range there was a predominance of NH_3^+ or COO^- ions depending upon the type of gelatin. Therefore, at these pH values, strength of electrostatic interactions could be maximized, resulting in small particles with low PDI [53]. It was found that increasing the bloom number of gelatin resulted in reduction in the particle size and PDI [49]. However, the increase in gelatin concentration and volume of ethanol led to an increase in the particle size [56]. Ethanol concentrations between 65 and 70% w/w yielded GNPs of small particle size and low PDI [10]. Azarmi et al. [57] showed that nanoparticles prepared with acetone as desolvating agent were generally smaller and of lower PDI compared to those prepared with ethanol.

Zeta potential is an important index for the stability of the GNP suspension. A high absolute value of zeta potential indicates high electric charge on the surface of the drug-loaded GNPs, which can cause strong repellent forces among particles to prevent their aggregation [3]. The positive charge on the surface of type A GNPs could be attributed to predominance of NH_3^+ groups, whereas the negative charge on the surface of type B GNPs could be attributed to a predominance of COO^-, acquired during the formulation of GNPs in acidic (pH 3) and basic (pH 11), respectively. Nahar et al. [49] noticed the relatively high zeta potential of GNP A bloom 300 over GNP A bloom 175 which could be explained by the higher molecular weight of the former and hence higher density of amine groups at the surface.

5.4. Cytotoxicity and cellular uptake

Cytotoxicity assays have been carried out to determine the effect of GNPs on the viability of the cells in culture. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple non-radioactive colorimetric assay to measure cell cytotoxicity, proliferation or viability. Using this standard MTT assay, Gupta et al. [17] demonstrated that human fibroblasts incubated with GNPs remained more than 100% viable at concentration as high as 500 μg/mL. Moreover, after incubating GNPs with human bronchial epithelial cells, the nanoparticles provoked little or no cytotoxicity observations as indicated by constant lactate dehydrogenase levels equal to the controls after 48 and 96 h [58]. Additionally, GNPs showed no inflammatory potential as they did not initiate any expression of interleukin-8 even at concentrations as high as 100 μg/mL [58]. These studies indicate that GNPs are biocompatible and non-toxic, and hence are safe to be used as a vehicle for drug delivery applications. However, when compared with rHG nanoparticles after incubation with human embryonic kidney cells, the cell viability of GNPs was slightly less than that of rHG nanoparticles suggesting that rHG nanoparticles may be more compatible as drug delivery systems for human applications [56].

Efficient cellular uptake in the disease area is a prerequisite for nanoparticles being used as a drug delivery system. The uptake of nanoparticles into cells is usually examined by confocal laser scan microscopy (CLSM) and flow cytometry (FACS). Type B GNPs loaded with the fluorescent macromolecular dye (tetramethylrhodamine-conjugated dextran, TMR–dextran) could be taken up by NIH-3T3 murine fibroblast cells through non-specific endocytosis and within 12 h, the payload could be released and accumulated around the perinuclear region [17]. Another study demonstrated that internalization of the fluorescent probe in GNPs by murine bone marrow dendritic cells (DCs) was higher (88%) than that of the soluble form of TMR–dextran (4%) [59]. GNPs were phagocytosed by DCs and were mostly localized in the lysosomes, with some escape into the cytoplasm, but no localization in the nucleus. However, Karthikeyan et al. [60] noticed that resveratrol-loaded GNPs exhibited very rapid and more efficient cellular uptake in lung cancer cells than free drug with fluorescence that was found inside the nuclei indicating that GNPs are useful in site-specific delivery of drugs to the cell nucleus. Many experiments indicated that GNPs were efficiently internalized and localized within various types of cells showing that these nanoparticles could be used for efficient intracellular delivery of biopharmaceuticals as well as for increasing drug delivery across cellular barriers.

5.5. Stability

The kinetic stability in biological media is an essential property of drug nanocarriers. The elevated stability of the drug-loaded particles may improve their blood circulation time [25]. Therefore, it is important to evaluate the physical stability of the colloidal GNP suspensions at relevant storage and physiologic temperatures at 4° and 37 °C, respectively. High storage stability of GNP suspensions over storage at 4 °C was reported as indicated by no significant differences in the size, zeta potential and PDI values up to 6 months [41,46]. However, a significant particle growth and reduced zeta potential of cryptolepine-loaded GNP suspension were noticed after just 2 weeks of storage at room temperature [61]. Thus, for long-term storage at room temperature, it may be appropriate to store GNPs in a freeze-dried state. In the study of Zillies et al. [62], oligonucleotide-loaded GNPs were successfully freeze-dried using trehalose as a cryoprotectant due to its high glass transition temperature and its amorphous nature. The freeze-dried GNP formulations retained biological activity *in vivo* after 4 weeks of storage at 40 °C. In another study, glucose and sucrose were found to be effective in low concentrations compared to mannitol as cryoprotectants in the preparation of freeze-dried cryptolepine-loaded GNPs. The particle size, zeta potential, PDI and drug release characteristics of lyophilized nanoparticles did not change after storage at 25 °C/60% relative humidity for 52 weeks [61].

6. Pharmaceutical applications of GNPs

6.1. Anti-cancer drug delivery

GNPs have been extensively used for the delivery of both hydrophilic and hydrophobic anti-cancer drugs including methotrexate [12], cytarabine [14], camptothecin [25], 17-AAG [26], curcumin [31], cycloheximide [53], resveratrol [60], doxorubicin (DOX) [15,28,34,55,63–68], paclitaxel [11,69], cisplatin [70–75] and noscapine [76], aiming to increase anti-tumor efficacy, control release, and target the drugs to the tumor thus reducing the toxicity. The benefits of GNPs for use in anticancer drug delivery include their very low cytotoxocity, simple and reproducible production that may lead to future upscaling and the low cost of gelatin [3]. Another important feature is the passive targeting ability of GNPs through the EPR effects, through which the nanoparticles remain at the tumor region for sufficient time to complete the release of the loaded anti-cancer drug which will be accumulated at the tumor region at high local concentration even with low doses and low frequency. These findings were confirmed by the rapid uptake and long-term retention demonstrated by GNPs in the tumor after administration. Paclitaxel-loaded GNPs showed 2.6 times higher bladder tumor tissue concentrations compared with the commercial Cremophor/EtOH formulation after intravesical administration into dogs [11].

The superior efficacy of anti-cancer drug-loaded GNPs compared to free drug was manifested both *in vitro* (in cancer cell lines) and *in vivo* (in tumor-bearing animal models). The tumor volume in the mice treated with free 17-AAG was increased 20 times relative to the initial volume, whereas that of 17-AAG/rHG-TOS nanoparticles was increased 15 times (Fig. 5) [26]. After intraperitoneal injection of DOX-loaded GA-crosslinked GNPs into rats, the efficiency of DOX was enhanced compared to free drug, however, high cardiotoxicity was observed upon repeated administration. The authors attributed this to covalent attachment of the drug to the carrier and the toxicity of the degradation products of drug–peptide conjugates [63]. According to Lee et al. [64], gelatin was conjugated with DOX after amine group-blockage using acetaldehyde. Gelatin–DOX nanoparticles exhibited much lower cytotoxicity and remarkably inhibited tumor growth and suppressed pulmonary metastasis compared to free DOX. GNPs loaded with paclitaxel were

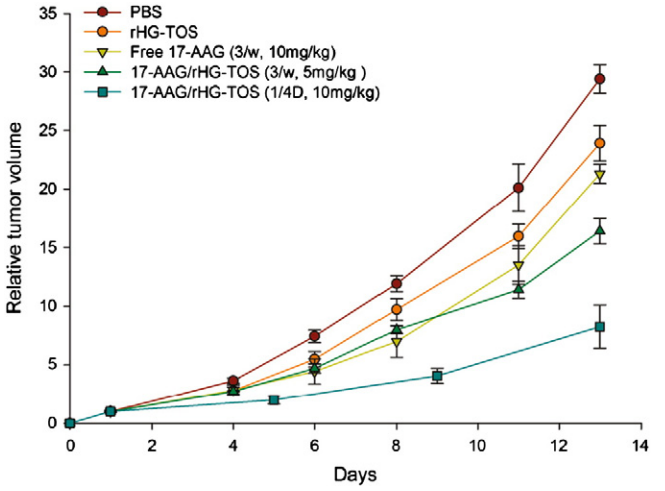

Fig. 5. Anticancer effects of 17-AAG-loaded rHG-TOS nanoparticles in tumor-bearing mice [26].

also used in intravesical bladder cancer therapy. Both the hydrophilicity of gelatin and the nanoencapsulation of paclitaxel in amorphous state within GNPs may be responsible for the enhanced solubility and rapid release of drug (87% in 2 h without enzymes) which is highly desirable in intravesical bladder therapy in which the drug formulation is typically maintained in the bladder for only a short duration (*i.e.*, 2 h) [11].

Cytotoxicity analysis of anti-cancer drug loaded GNPs against different cancer cell lines showed the higher anti-cancer activity compared to free drug which might be attributed to greater endocytotic uptake of GNPs in cancer cells. The IC_{50} value of noscapine-loaded GNPs on human breast cancer cell line (MCF7) was 26.3 μM significantly lower than that of free noscapine (40.5 μM) [76]. Similarly, encapsulation of resveratrol into GNPs enhanced its anti-cancer efficacy against NCI-H460 lung cancer cells compared to free drug [60]. Erythrocyte aggregation assay showed that the prepared resveratrol-GNPs elicited no hemolytic response whereas the free drug caused a significant aggregation of erythrocytes upon 1 h incubation.

6.2. Protein and vaccine delivery

GNPs have also been used to deliver protein and peptide drugs. Li et al. [77,78] have studied GNPs for the delivery of BSA as a model protein. A composite system composed of BSA-loaded GNPs encapsulated in poly(lactic-co-glycolic acid) microspheres demonstrated sustained release characteristics with the capability of preventing protein denaturation [77]. In another study, rHG nanoparticles showed great potential for delivery of FITC-BSA in terms of sustained release, less initial burst, and safety [41]. Other protein drugs including insulin [16], tissue-type plasminogen activator (t-PA) [79,80], bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP) [4] and angiogenic basic fibroblast growth factor (bFGF) [81] were successfully encapsulated into GNPs with the biological activity of those protein drugs were retained *in vivo*.

Significant uptake of GNPs by murine bone marrow dendritic cells (DCs) has been reported and therefore, they are suitable for targeting antigens to DCs and can be a suitable immunoadjuvant [59]. Subcutaneous injection of tetanus toxoid (TT)-loaded GNPs in BALB/c mice effectively elicited systemic immune response as demonstrated by comparable IgG response and a significantly higher cytokine response (IL-2 and IFNγ) as compared to alum-TT vaccine [82]. After uptake by antigen-presenting cells (APCs), GNPs are degraded by lysosomal enzymes (*e.g.* collagenase) releasing TT intracellularly. Moreover, GNPs could deliver a large amount of antigen to APCs, leading to an antigen specific immune response.

6.3. Gene delivery

GNPs have several advantages as a non-viral gene delivery vector. They can be conjugated to moieties that stimulate receptor-mediated endocytosis, multiple plasmids can be encapsulated and the bioactivity of the encapsulated DNA could be improved by preventing digestion by nucleases and by using long-circulating PEGylated nanoparticles [3,83]. Nucleic acids can be loaded onto GNPs through physical encapsulation, electrostatic attraction or complexation with surface modifying groups. Kaul and Amiji [54] were the first to develop type B GNPs as non-condensing gene delivery systems. The negatively charged type B gelatin at neutral pH 7.0, can physically encapsulate reporter and therapeutic nucleic acid constructs as opposed to positively charged lipids and polymers that electrostatically condense DNA. The physically encapsulated plasmid DNA (pDNA) in a hydrogel-type matrix is protected in the systemic circulation and upon cellular transport. Additionally, the released pDNA has a supercoiled structure at the nuclear membrane which is critical for efficient uptake and transfection [84]. In other instances, the negatively charged nucleic acids can be adsorbed onto the surface of GNPs by modifying the surface of gelatin with a quaternary amine (*e.g.* cholamine) to increase ionic interactions [85]. Another method of loading nucleic acids was reported through complexation of the antisense-compound namely biotinylated peptide nucleic acid (PNA) by the avidin-conjugated GNPs [86].

Over the last several years, Amiji group has investigated the potential of non-condensing type B GNPs for systemic and oral gene therapy. Cell trafficking studies using GNPs loaded with TMR–dextran as a model hydrophilic drug in BT-20 cells showed that the particles were mainly taken up by endocytosis, which later escaped the endosomal system and were found around the perinuclear area in the cytoplasm [54,87]. In another study, most of the administered GNPs were internalized in NIH-3T3 fibroblast cells within the first 6 h of incubation. Green fluorescent protein expression was observed after 12 h of nanoparticle incubation and remained stable for up to 96 h with DNA transfection efficiency was 43% [88]. Leong et al. [89] have studied the *in vivo* transfection efficiency of GNPs containing the LacZ plasmid in the tibialis anterior muscle of 6-week-old BALB/c mice. The GNPs exhibited a more profound and sustained gene expression than the naked pDNA and lipofectamine complexes. GNPs were also used for successful encapsulation and intracellular delivery of siRNA [90]. The encapsulated siRNA was shown to be stable even in RNAse rich environment. Following treatment of HIF-1α siRNA loaded GNPs in HIF-1α overexpressed SKOV3 cells, it has demonstrated significant down-regulation of HIF-1α [90,91].

Bhavsar and Amiji [92–94] developed a unique multicompartmental oral DNA delivery system based on encapsulation of DNA-loaded type B GNPs in poly(epsilon-caprolactone) (PCL) microspheres using a "double emulsion-like" technique. This delivery system was termed "nanoparticles-in-microsphere oral system" or NiMOS (Fig. 6). NiMOS would be able to protect the orally administered nucleic acids during transit from the stomach. When PCL matrix is degraded by lipases abundantly present in the intestinal tract, the released DNA-containing GNPs can then be internalized by the enterocytes or other cells of the GI lumen for transfection of the encoded protein [92–95].

Following oral administration of reporter pDNA encoding enhanced green fluorescent protein (EGFP-N1) or beta-galactosidase (CMV-βgal) in NiMOS less than 5.0 μm in diameter to fasted Sprague–Dawley rats, there was significant EGFP and beta-galactosidase expression in the small and large intestine [94]. After oral administration of anti-inflammatory murine IL-10 (mIL-10) expressing pDNA-loaded NiMOS

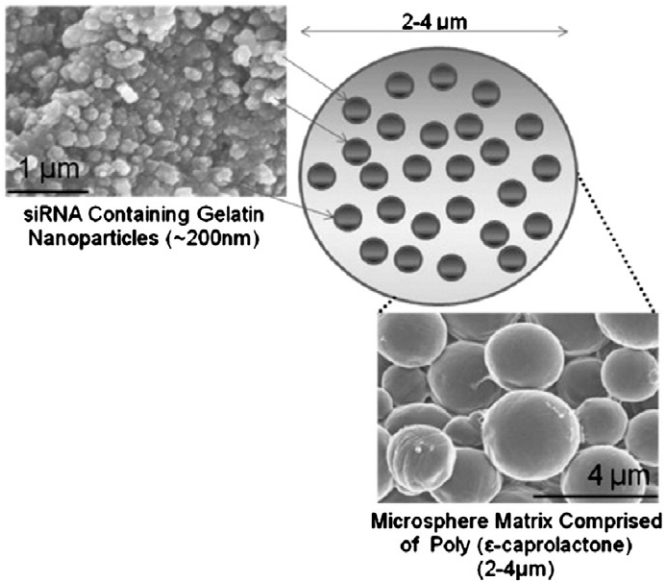

Fig. 6. Schematic representation of the NiMOS and the scanning electron micrographs of small interfering RNA (siRNA)-encapsulated type B GNPs and siRNA containing NiMOS [97].

into Balb/c mice with induced colitis, the mice showed significantly higher mRNA and protein levels in addition to reduced levels of pro-inflammatory cytokines such as IL-1a, IL-1b, IFN-γ, TNF-α, and IL-12 as compared to mIL-10 pDNA in naked GNPs [96]. Kriegel and Amiji [97] have recently extended the application of NiMOS for oral delivery of siRNA duplexes for treatment of inflammatory bowel disease. After oral administration of TNF-α specific siRNA encapsulated NiMOS in mice, successful gene silencing led to decreased colonic levels of TNF-α, suppressed expression of other pro-inflammatory cytokines (*e.g.*, IL-1β, IFN-γ) and chemokines (MCP-1), an increase in body weight, and reduced tissue myeloperoxidase activity.

6.4. Ocular drug delivery

GNPs were chosen for ocular drug delivery because of its biocompatibility and biodegradability. Moreover, collagen, the native protein from which gelatin is derived, is present in the eye, more specifically in the stroma of the cornea, and has been extensively employed in ocular applications [98]. GNPs encapsulating hydrophilic (pilocarpine HCl) and hydrophobic (hydrocortisone) drugs were produced for topical ophthalmic use [51]. Uptake of those nanoparticles in the first cell layers of the cornea was expected dependent on their nanometric size. Sustained release of both drugs from GNPs was observed with the release kinetics were close to zero order [51]. Cationized GNPs have been successfully used for transfecting the ocular epithelium offering advantages of protection of pDNA and increased transfection efficacy. Spermine-cationized GNPs were used to efficiently transfect human corneal epithelial (HCE) cells *in vitro* [99]. The nanoparticles significantly protected the associated pDNA from degradation in the presence of DNase I for at least 60 min compared to 5 min for naked DNA to be completely digested. The confocal images of HCE cells showed effective internalization of the pDNA by nanoparticles with the absence of naked pDNA internalization [99]. The nanoparticles successfully transfected the ocular epithelial cells *in vitro* and *in vivo* with a new plasmid encoding a modified human MUC5AC mucin protein [100]. Corneal cell lines showed detectable MUC5AC mRNA expression in cells exposed to the nanoparticles. The *in vivo* application of the pMUC5AC-loaded GNPs onto the eyes of rabbits resulted in significantly higher MUC5AC expression in the conjunctiva (122%) compared to untreated control and naked plasmid.

6.5. Pulmonary drug delivery

GNPs may be promising vehicles of transporting drugs efficiently to the lung *via* inhalation, while avoiding unwanted mucociliary clearance. Tseng et al. [70] reported that inhalable GNPs do not cause lung inflammation as demonstrated by myeloperoxidase activity assay and are thus safe for use. Therefore, inhalative GNPs as carriers of cisplatin were designed for the treatment of lung cancer with anticipated improved therapeutic effect and reduced side effects. A high cisplatin concentration could be achieved in cancerous lungs *via* inhaled GNPs [70]. However, the disadvantage of using nano-sized delivery systems for pulmonary application is that their mass median aerodynamic diameter (MMAD) is not suitable for inhalation purposes. Consequently, it is expected that a large fraction of the inhaled dose will be exhaled and little particle deposition will take place in the lungs. To overcome this problem, Sham et al. [101] investigated spray-dried micrometer-sized carrier particles for delivery of GNPs to the lower respiratory regions of the lung *via* a dry powder inhaler. GNPs were spray-dried together with lactose as the carrier matrix which is expected to dissolve quickly after landing on the aqueous covered epithelium of the lung and nanoparticles are released immediately. The mean particle size of spray-dried GNPs increased from 242.2 to 319.9 nm; however, the fine particle fraction (FPF 40%) and MMAD (3.0 μm) of the powders were suitable for efficient lung delivery [101].

An aerosol formulation of cationized GNPs loaded with immunostimulating agent; Cytosine–Phosphate–Guanine–Oligodeoxynucleotides (CpG–ODN) was developed to maximize the efficacy of immunotherapy against hypersensitivity for treatment of equine recurrent airway obstruction [102,103]. The size of GNPs after nebulization was 222.3–248.2 nm, with a FPF of up to 65.7%. Nebulized CpG–ODN-loaded GNPs remained capable to stimulate IL-10 release *in vitro* from equine alveolar lymphocytes. After five consecutive inhalations, regulatory anti-inflammatory and anti-allergic cytokine IL-10 expression was significantly triggered. Recently, GNPs were investigated as insulin pulmonary administration system [16]. Intratracheal instillation of insulin-loaded GNPs was found to promote insulin pulmonary absorption effectively and prolong the duration of hypoglycemic effect. In addition, nanoparticles could guarantee the safety of lung by reducing insulin deposition in lung.

6.6. Nutraceutical delivery

Over recent years, GNPs hold promise for efficient delivery of several bioactive nutraceuticals with the aim of improving their poor bioavailability, enhancing their stability and bioactivity in addition to providing controlled release. According to Chen et al. [29], the antioxidant activity of tea catechins was retained after three weeks of storage *via* encapsulation into GNPs. In addition, tea catechins exhibited 28–41% inhibition to trypsin against the degradation of gelatin. Thus, tea catechin-GNPs might be a useful antioxidant carrier because catechins and gelatin were, respectively, protected from oxidation and enzymatic digestion.

Natural polyphenols, epigallocatechin gallate (EGCG), tannic acid, curcumin, and theaflavin were encapsulated into LbL coated GNPs with the aim of improving their poor bioavailability and half-life prolongation [32]. Western blot analysis showed that GNP-encapsulated EGCG retained its biological activity by blocking hepatocyte growth factor (HGF)-induced intracellular signaling in breast cancer cell line as potently as free EGCG. In another study, gelatin–dextran micelles exhibited high loading, and sustained release of tea polyphenol (TPP) *in vitro* and showed stronger cytotoxicity against breast cancer cells than free TPP [42]. Nanoencapsulation of cocoa procyanidins (CPs) into GNPs significantly improved their storage stability and enhanced their apoptotic activity in human acute monocytic leukemia cells compared with the free CPs [104]. Moreover, coating the nanoparticles with chitosan imparted positive charge to facilitate electrostatic interaction with the negatively charged mucus layers, which favors the transportation of nanoparticles to the epithelium. The authors attributed the observed stabilizing effect to the interaction between gelatin and CPs, the protection of chitosan, and the restricted diffusion of O_2 through the polymer barrier into the nanoparticle space.

6.7. Enzyme immobilization

GA-crosslinked GNPs were successfully used for glucoamylase immobilization [105]. Based on the reversible swelling and contracting of the crosslinked GNPs with several cycles of heating and cooling, this system could be used for enzyme immobilization and release. The immobilized enzyme was released when the system temperature was above 40 °C and performed high activity similar to free enzyme. On the other hand, when the system temperature was below 40 °C, there was no enzyme release. The loading efficiencies of glucoamylase immobilized by entrapment and adsorption methods were 59.9% and 24.7%, respectively. The efficiency of temperature-triggered release was as high as 99.3% for adsorption method, while the release of enzyme from the entrapment method was not detected. The authors attributed this to the possible reaction of GA with glucoamylase forming covalent linkage between gelatin and glucoamylase resulting in the higher immobilization efficiency for entrapment method. However, the covalently linked enzyme was hardly to be released. On the other

hand, in adsorption method, the glucoamylase was only physically adsorbed at the gelatin matrix which facilitated its release [105].

6.8. Miscellaneous drug delivery

GNPs have been also utilized as delivery systems for drugs of miscellaneous classes including anti-HIV (didanosine [106]), anti-malarial (chloroquine phosphate [13] and cryptolepine hydrochloride [61,107]), anti-fungal (fluconazole [20] and amphotericin B [49,108,109]), anti-tubercular (rifampicin [110] and isoniazid [111]), anti-bacterial (gatifloxacin [20], chloramphenicol [47] and ciprofloxacin hydrochloride [112]), anti-inflammatory (ibuprofen [48,113] and indomethacin [114]), analgesic (paracetamol [43]), skeletal muscle relaxant (tizanidine hydrochloride [20]) and oral hypoglycemic (rosiglitazone [115]) drugs.

In those studies, GNPs were utilized for: *(i) providing prolonged drug release;* GNPs were found to be capable of releasing cryptolepine and ciprofloxacin hydrochloride for prolonged durations up to 192 and 96 h, respectively [61,112], *(ii) reducing the side effects of toxic drugs;* encapsulation of amphotericin B and cryptolepine into GNPs was found to reduce their nephrotoxic and hemolytic side effects, respectively, compared to the free compounds [49,107], and *(iii) improving the pharmacokinetic profile and pharmacological activity of drugs;* after i.v. injection into Wistar rats, cryptolepine-loaded GNPs attained a 4.5 fold higher area under the curve and longer elimination half-life (21.85 h) compared to free drug (11.7 h) in addition to improved *in vivo* schizonticidal activity [107]. Rifampicin-loaded GNPs resulted in enhanced uptake of drug by the lung tissue, improving its bioavailability causing significant reduction in bacterial counts in the lungs and spleen of TB-infected mice [110]. Similarly, the anti-inflammatory activity of indomethacin-loaded GNPs was enhanced compared to pure drug as indicated by the significant decrease in the rat paw volume [114].

7. Surface-modified GNPs

The primary structure of gelatin offers many possibilities for chemical modification. When gelatin surface was modified with site-specific ligands, its uptake was further facilitated by receptor-mediated endocytosis [3].

7.1. Thiolated GNPs

The intracellular glutathione (GSH) concentration is generally higher than the extracellular concentrations. While during active proliferation of tumor cells, GSH and peroxide levels are even higher in the cytoplasm. Therefore, Kommareddy and Amiji [116] introduced thiol (*i.e.*, SH) groups into gelatin through reaction with 2-iminothiolane then thiolated type B GNPs were prepared by desolvation for cytosolic DNA delivery in response to higher intracellular GSH concentrations. Thiolated GNPs showed a greater percent release of fluorescein isothiocyanate-dextran in GSH containing phosphate buffer (pH 7.4) as compared to unmodified GNPs [116]. The thiol content of gelatin would result in the formation of disulfide bonds within the polymer structure, thus strengthening the tertiary and quaternary protein structure in the case of gelatin. The disulfide bonds also stabilize the nanoparticles during systemic circulation. However, in the cell, where the GSH concentrations are usually 1000 fold higher, these disulfide bonds are broken and the biopolymer unfolds releasing its contents [116,117]. Thiolated GNPs encapsulating pDNA encoding for EGFPN1 were found to have greater transfection efficiency in NIH-3T3 murine fibroblast cells as compared to unmodified GNPs and Lipofectin®-complexed DNA. The high transfection efficiency associated with thiolated GNPs could be attributed to the increased stability from additional crosslinking, noncomplexed DNA delivery system, trigger release of the payload in a reducing environment inside the cell, and reduced cytotoxicity.

7.2. Pegylated GNPs

GNPs are predominantly engulfed by the cells of RES upon systemic administration. With coating of poly(ethylene glycol) (PEG), it could form a dense hydrophilic shell of long chains and protect the core from non-specific hydrophobic interaction with serum proteins (*e.g.*, opsonins) resulting in reduced uptake by RES [118]. Furthermore, PEGylation may also increase the hydrodynamic size of the particles decreasing their clearance through the kidney. This would ultimately result in an increase in circulation half-life of the particles *in vivo* [119]. Moreover, chemical derivatization of PEG through its terminal hydroxyl group can be used for site directed PEGylation of protein or for further conjugation of selected ligands. Additionally, the presence of PEG chains on the surface of GNPs was found to resist digestion by proteolytic enzymes [90]. A gelatin PEGylation process by reacting gelatin and PEG-epoxide was illustrated in Fig. 7.

Combined with the high transfection ability of non-condensing type B GNPs, PEGylated GNPs are preferentially distributed to solid tumor due to the hyperpermeability of the angiogenic blood vessels by the EPR [120,121]. Thus, PEGylated non-condensing type B GNPs are suitable for systemic gene delivery to tumor mass. In the study of Kaul and Amiji [120], PEGylated GNPs were shown to preferentially target the tumor mass in Lewis lung carcinoma (LLC) bearing female mice and approximately 4–5% of the intravenously injected dose remained in the tumor for up to 12 h postadministration. In the later study from the same group, reporter pDNA encoding for β-galactosidase (pCMV-β) was encapsulated in PEGylated GNPs [121]. Intravenously administered PEGylated GNPs to LLC-bearing mice showed significant expression of β-galactosidase in the tumor with 61% transfection efficiency relative to i.t. administration. Kushibiki et al. [122] have proven the long-circulation property of PEGylated gelatin by using ^{125}I-labeled gelatin. The authors further examined the biodistribution profiles of unmodified and PEGylated ^{125}I-labeled GNPs following i.v. administration through the tail vein in LLC-bearing mice. PEGylated nanoparticles showed long circulating properties in the blood and preferentially accumulated in the tumor for up to 24 h post-administration. In another study, thiolated PEGylated GNPs showed prolonged circulation times and enhanced tumor extravasation *in vivo* in an orthotopic human breast adenocarcinoma xenograft model [123]. The nanoparticles were found to have longer circulation times non-PEGylated ones, with the plasma and tumor half-lives of 15.3 and 37.8 h, respectively.

In addition to gene delivery, PEGylated GNPs have been also used as long circulating passive targeting system for efficient delivery of various anti-cancer drugs to tumors. PEGylated gelatin–DOX nanoparticles significantly inhibited tumor growth up to 82% and were systemically

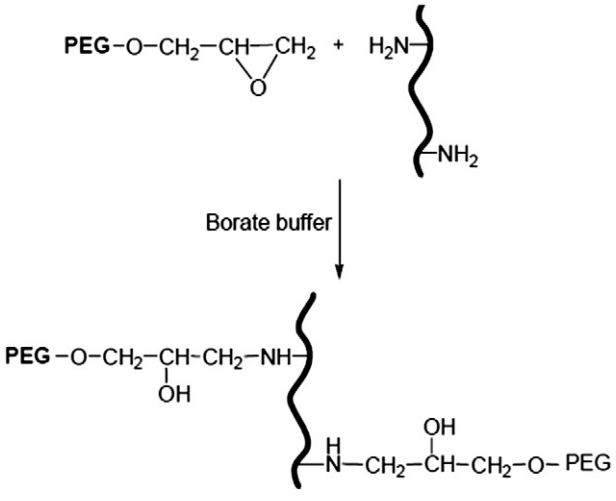

Fig. 7. A scheme for the synthesis of PEGylated gelatin [64].

less toxic than DOX based on hematological, serological and histopathological findings [64]. I.V. administration of noscapine, the tubulin-binding anticancer agent, resulted in rapid elimination of the drug with a half-life of 0.39 h [76]. A 1.43-fold increase in the area under the curve for noscapine-loaded PEGylated GNPs over unmodified GNPs and a 13.09-fold increase over free noscapine was noted with significantly enhanced cytotoxicity. Similarly, PEGylated gelatin-polylactic acid nanoparticles improved the phototoxic efficacy of cyclohexane-1,2-diamino hypocrellin B, against human breast adenocarcinoma (MCF-7), human gastric sarcoma (AGS) and mice specific Dalton's lymphoma (DLA) compared to free drug [124]. Recently, ibuprofen sodium (IbS) was encapsulated into PEGylated GNPs for injection to overcome its limited bioavailability and rapid clearance thereby aiding reduction in its administration frequency [48]. The nanoparticles provided sustained drug release for about 4 days with improved bioavailability and plasma half-life when compared to non-PEGylated GNPs and free IbS. Histological analysis of liver and kidney revealed tissue integrity indicating biocompatibility of the nanoparticles.

7.3. Cationized GNPs

Gelatin is a low charge density polyelectrolyte with the net charge appreciably dependent on solution pH. Of particular interest is the modification of the gelatin charge by cationization [125]. Cationic gelatin can be readily prepared by simply introducing amine residues to the carboxyl groups of gelatin *via* reaction with ethylenediamine [79,80,126,127], cholamine [85,102,103], polyethylenimine [128,129] and spermine [99,100]. Cationized GNPs have been used for a myriad of applications that benefit from the increased positive charge on the GNPs.

As delivery vehicles for pDNA, positively charged GNPs could be capable of condensing DNA and favoring interactions with the negatively charged cell membrane to facilitate endocytosis [126]. There was five-fold elevation in the amount of insulin-like growth factor (IGF)-1 produced by the adult canine articular chondrocyte cells treated with the ethylenediamine cationized GNPs containing the pIGF-1 compared with the noncationized GNPs. A higher positive surface charge may enable to condense the pIGF-1 producing smaller nanometer-sized particles that could increase cell entry. Once inside the cells, the nanoparticles would release the plasmid, which would gain entry into the nucleus and enable the overexpression of IGF-1 [126]. Polyethylenimine (PEI) has high positive charge and shown high affinity to DNA through the electrostatic interaction between the amino group of PEI and phosphate groups of the DNA. Moreover, PEI has high endosomal and lysosomal-buffering capacity as a "proton sponge" which can protect the nucleotides from degradation and promote their release from the acidic vesicles [128]. Therefore, low molecular-weight PEI was conjugated onto the surface of GNPs as delivery vector for DNA of pCMV-Luc gene. The nanoparticles provided high transfection efficiency with minimal cytotoxicity.

Cationized GNPs could be also used to develop a sustained release system of small interfering RNA (siRNA) inside cells aiming at a prolonged time period of gene suppression. Ethylenediamine cationized GNPs succeeded to prolong the release of luciferase siRNA with the time profiles of siRNA release corresponded to those of nanospheres degradation [127]. In another study, novel core–shell nanocarriers composed of a biodegradable gelatin core and a highly extended PEI layer as a shell were developed for siRNA delivery [129]. Gelatin-PEI nanogels were able to completely condense siRNA, and effectively protected siRNA against enzymatic degradation. The nanogels were able to effectively deliver siRNA into HeLa cells increasing the intracellular uptake efficiency of siRNA from 41 to 84% with the delivered siRNA could inhibit 70% of human argininosuccinate synthetase 1 gene expression.

According to reports in the literature, positively charged particles are favorable phagocytosed by DCs and macrophages compared to neutral or negatively charged particulate formulations [59]. In agreement with this, murine myeloid DCs internalized the positively charged immunostimulatory CpG ODN-loaded cholamine cationized-GNPs more efficiently than plain non-cationized GNPs, which have a partially negative zeta potential [85]. Surface modification with cholamine hydrochloride resulted in the formation of a pH-independent cationic surface charge on the nanoparticles, which prevent unwanted desorption of the CpG ODN from the carrier surface during the transport to the target cell.

The positive zeta potential seems to be very important for improving the interaction between GNPs and the ocular surface leading to a higher transfection efficiency of the system [130]. Gelatin cationized with the low molecular polyamine spermine was the most promising among the studied cationized proteins in terms of safety, formation of nanoparticles with suitable physicochemical properties, protection of pDNA, and transfection efficiency in human corneal cells. Zorzi et al. [99] developed hybrid nanoparticles composed of spermine cationized gelatin and the polyanions, dextran sulfate and chondroitin sulfate, using mild ionic gelation technique to induce gene expression in ocular epithelial cells. Such anionic polymers have the ability to interact with the hyaluronic acid receptor for endocytosis (HARE) and CD44, both of which are found in the eye and related to internalization of macromolecules [131].

Cationized GNPs could be used for complexation with anionic drugs rather than nucleic acids. Ethylenediamine-cationized gelatin electrostatically interacted with tissue-type plasminogen activator (t-PA), an anionic globular protein widely used as a thrombolytic drug, forming cationized t-PA–gelatin complex which upon simple mixing with the anionic PEGylated-type B gelatin forms a nano-sized delivery complex *via* electrostatic attraction [79]. The t-PA thrombolytic activity of complexes was significantly suppressed to be 45% of original t-PA. However, when exposed to ultrasound *in vitro*, the t-PA activity was fully recovered. In a rabbit thrombosis model, the i.v. administration of the complexes followed by ultrasound irradiation resulted in complete recanalization. In another study, the interaction between t-PA and cationized gelatin molecules could be reinforced by mixing with zinc ions. t-PA molecules electrostatically interact with gelatin to form their complex, and additionally, the complex becomes stable through coordination bond with zinc ions. The t-PA complexation with gelatin and zinc ions enabled to prolong the half-life of t-PA in the blood circulation because the apparent molecular size of t-PA was reduced to 95 nm [80].

7.4. Antibody-anchored GNPs

Antibody modified GNPs have been used as drug carrier systems to target nanoparticles to specific cell types. In the work of Balthasar et al. [132], the surface of GNPs was thiolated and the avidin derivative NeutrAvidin™ was covalently attached to the nanoparticles *via* bifunctional spacer (Fig. 8). Due to its high binding affinity for biotin, biotinylated compounds such as antibodies can be very rapidly attached by strong avidin–biotin complex formation. Thus, biotinylated anti-CD3 antibodies specific for the CD3 antigen on lymphocytic cells were attached to GNPs for specific drug targeting to T-lymphocytes [132,133]. In cell culture, these nanoparticles very specifically bound to CD3+ human T-cell leukemia cells and primary T-lymphocytes. Uptake rates of about 84% into T-cell leukemia cells were observed. The cell-type specificity was further confirmed by competition experiments using excessive free anti-CD3 antibodies which could suppress binding and internalization of anti-CD3-modified nanoparticles. The uptake mechanism could be characterized as a receptor-mediated endocytosis by incubation at 4 °C or in the presence of cytochalasin which inhibits the uptake of αCD3-nanoparticles [133].

7.5. Peptide-conjugated GNPs

Peptide ligands, which have the advantage of high avidity of interaction with the target receptor through multiple points of contact, low

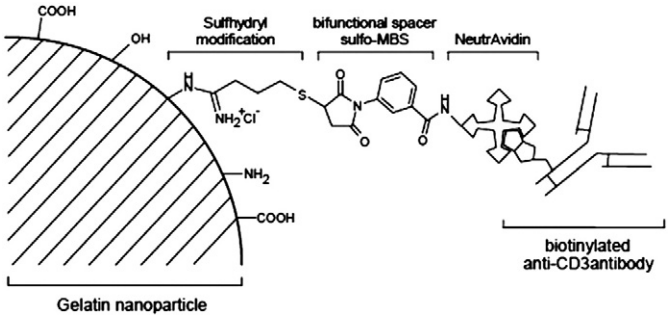

Fig. 8. Schematic representation of antibody-anchored NeutrAvidin™-modified GNPs [132].

immunogenicity, and easier surface modification of the nanocarrier systems, are being pursued by a number of groups as targeting moiety for cell-specific delivery [134]. Overexpression of epidermal growth factor receptors (EGFR) has been observed to correlate with poor prognosis in several types of cancers. Thus, conjugation with EGFR targeting peptide should assist the system in active targeting of tumor cells. The EGFR-targeted GNPs carrying pDNA encoding for EGFP-N1 showed highest levels of EGFP expression in Panc-1 pancreatic adenocarcinoma cells relative to all the other controls, especially at 48 h posttransfection [84]. With the same system, transfection with tumor suppressor protein (p53) induced rapid apoptosis process in Panc-1 cells [135]. After intravenously injected into mice bearing Panc-1 human pancreatic adenocarcinoma, EGFR-targeted GNPs showed preferential and sustained accumulation in the tumor mass, and displayed almost twice tumor targeting efficiency than either PEGylated or unmodified nanoparticles, highlighting the efficacy of the active targeting strategy [136]. Similarly, EGF-conjugated gelatin–cisplatin nanoparticles resulted in higher cisplatin concentrations in lung adenocarcinoma cells (A549, high EGFR expression) than that on normal lung cells (HFL1, low EGFR expression). Nebulized aerosol droplets of EGF-GNPs showed specific accumulation in the cancerous lung of mice to achieve high cisplatin dosage with stronger anti-tumor activity and lower toxicity [70,137].

Tat and SynB peptides are cell-penetrating peptides that show charge-mediated blood–brain barrier (BBB) selectivity [138,139]. Therefore, modification of gelatin–siloxane nanoparticles (GSNPs) with the Tat or SynB peptide enhanced their efficiency in crossing BBB. Membrane penetration is driven primarily by ionic interaction between the cationic charges of these peptides and the anionic charges of the phospholipid heads in the biomembrane, and subsequently internalized into cells by endocytosis [140,141]. When the plasmid encoding calcitonin gene-related peptide (pLXSN-CGRP) was encapsulated into Tat-GSNPs, 1.71 and 6.92 times higher CGRP expression in endothelial cells than unmodified GSNPs and naked pLXSN-CGRP, respectively were observed [142]. Because of the effect of the nuclear localization signal in the Tat peptide, pLXSN-CGRP could be transferred into the nucleus, and then released from the nanoparticles for efficient gene transfection. After intracisternal injection of (pLXSN-CGRP)-loaded Tat-GSNPs in an experimental rat model of subarachnoid hemorrhage, overexpression of CGRP attenuated vasospasm and improved neurological outcomes.

7.6. Carbohydrate-decorated GNPs

Macrophages possess variety of surface receptors such as mannosyl, lectin and galactosyl called mannose receptors (MRs). MRs are present at the surface of monocyte macrophages, alveolar macrophages, astrocytes in brain, hepatocytes in liver, and so on and overexpressed in infected macrophages [143]. Due to this fact, carriers containing ligands such as mannosyl, immunoglobulin, fibronectin, and galactosyl are better phagocytosed by macrophages. Therefore, GNPs bearing the anti-HIV drug didanosine were coupled with mannose for controlled and site-specific delivery to HIV-infected macrophages for improving its therapeutic efficacy and reducing its toxicity [106]. The coupling method involved ring opening of mannose followed by reaction of its aldehyde group with free amino groups of GNPs *via* Schiff's base formation. Coupling of mannose to GNPs enhanced their *in vitro* uptake in the macrophage tissues and so enhanced the lung, liver, and lymph nodes uptake of drug, in comparison to noncoupled GNPs or free drug. Similarly, mannosylated GNPs showed a 5.4-fold reduction in IC_{50} of amphotericin B in comparison with plain drug suggesting significant enhancement of its anti-leishmanial activity on J774A.1 infected macrophage cells with [108]. Saraogi et al. [111] found that incorporation of isoniazid into mannosylated GNPs enhanced its delivery to alveolar tissues resulting in significant reduction in bacterial counts in the lungs and spleen of TB-infected mice beside reduction in the drug hepatotoxicity.

Another ligand, 4-sulfated N-acetyl galactosamine (4-SO_4GalNAc), is closely related to the macrophage MRs both antigenically and structurally [65]. Cys-MR domain binds glycoprotein's bearing sulfated sugars by hydrogen bonding between sulfate group of 4-SO_4GalNAc and cystein group on MRs. The surface of GNPs was modified with 4-SO_4GalNAc for specific targeting of DOX to macrophages of liver and spleen in treatment of visceral leishmaniasis. After i.v. administration, the modified GNPs showed enhanced internalization in macrophage cells with significantly higher localization of DOX in liver and spleen as compared to unmodified ones. Another important application of carbohydrate-decorated nanovectors is the targeting of hepatocarcinoma cells [144]. Asialoglycoprotein (ASGP) receptors are present on liver cells and bind ASGPs (glycoproteins from which sialic acid has been removed to expose galactose) to remove them from circulation [145]. Galactosylated GNPs were prepared for enhanced intracellular delivery of DOX to hepatocarcinoma cells [66]. Galactosylated GNPs were selectively taken up by HepG2 cells and exhibited enhanced cytotoxicity (IC_{50}; 0.35 μg/mL) and apoptosis compared with unmodified GNPs (IC_{50}; 0.75 μg/mL) with liver accumulation of 24.5 μg/g DOX in liver in comparison with heart (0.3 μg/g) [66].

Heparin, a highly sulfated anionic polysaccharide composed of repeating glucosamine and uronic acid residues, interacts with a variety of growth factor receptors having heparin-binding domains such as fibroblast growth factor-2 (FGF2) receptors overexpressed on tumor cells including breast cancer cells [146]. When heparin was attached to cisplatin-bearing GNPs through amide bond between –COOH groups of heparin and $-NH_2$ groups of gelatin, a greater uptake into human breast cancer cells and greater tumor accumulation in tumor-bearing mice was exhibited compared to unmodified GNPs and free drug [71].

7.7. Fatty acid polymer-coated GNPs

In the study of Sarkar [147], a new kind of surface modification of GNPs rather than covalent attachment has been done with fatty acid polymers. Rhodamine B-encapsulated GNPs have been prepared by coacervation-phase separation then the fatty acids, myristoleic or oleic acid, have been polymerized *in situ* on the surface of gelatin coacervates

forming protective shells thereby retarding the dye release. It was found that GNPs coated with oleic acid have smaller size, greater dye encapsulation efficiency and slower release compared to that with myristoleic acid. The authors attributed this behavior to that the longer chain fatty acid, *i.e.*, oleic acid provides a more hydrophobic environment and helps protecting the dye more efficiently than that formed from a shorter chain fatty acid (myristoleic acid). The fatty acids can block the pores on the surface of GNPs and thus delay the release of encapsulated dye.

8. Gelatin nanocomplexes

8.1. Gelatin–polymer nanocomplexes

Gelatin has the ability to form nanocomplexes with different polymers through various mechanisms including ionic complexation, graft copolymerization or Maillard reaction. Injectable and biodegradable colloidal gels have been developed by mixing equal weight percents of oppositely charged cationic type A GNPs and anionic type B GNPs formed using a desolvation method [148]. These nanosphere-based gels were shown to be cohesive and self-healing due to the strong electrostatic interactions between cationic and anionic nanospheres and showed great potential for sustained delivery of osteogenic protein growth factors (BMP-2 and ALP) [4]. In another study, sequential release characterized by rapid release of angiogenic basic fibroblast growth factor (bFGF) and more sustained release of BMP-2 was obtained by loading bFGF onto cationic GNPs of low crosslinking density and BMP-2 onto anionic GNPs of high crosslinking density. Using *in vivo* rat femoral condyle defect model, an obvious stimulatory effect on bone regeneration was observed for the colloidal gels loaded with BMP-2, whereas bFGF-loaded colloidal gels did not influence the rate of bone regeneration [81].

Gelatin-polyacrylic acid (GEL-PAA) core–shell nanoparticles were prepared *via* polymerization of anionic acrylic acid monomers in the presence of cationic type-B gelatin without any aid of organic solvents or surfactants [72,149]. Driven by hydrogen bonding between unionized carboxyl groups of PAA and carbonyl groups of gelatin and electrostatic interactions between ionized carboxyl groups of PAA and protonated amino groups of gelatin, GEL-PAA nanoparticles were formed. Cisplatin was successfully loaded into GEL-PAA nanoparticles through a ligand exchange reaction of platinum(II) from the chloride to the carboxyl group of the nanoparticles. Furthermore, the abundant carboxylic groups provided by PAA dramatically enhanced cisplatin loading (24.6%) [72,73]. The nanoparticles showed significantly superior anticancer efficacy in hepatic H22 tumor-bearing mice in comparison with free drug. Cisplatin-loaded GEL-PAA nanoparticles showed prominent passive tumor-targeting ability and were able to penetrate the tumor after their extravasation through the leaky vessels and distributed in a distance of about 20 μm from the vessels at 24 h postinjection [73]. Upon peritumoral implantation of gelatin hydrogel (jelly) encapsulating cisplatin-loaded GEL-PAA nanoparticles in a murine hepatoma H22 cancer model, a significantly superior efficacy in impeding tumor growth and prolonging the lifetime of mice than those treated with i.v. injection of drug-loaded nanoparticles was observed [74]. The jelly maintains its hydrogel state while coating the tumor, then, gradually transforms into a viscous sol due to the body temperature, subsequently releasing the drug-loaded nanoparticles resulting in higher concentration, retention and penetration of drug in the tumor as well as lower uptake in nontarget organs (Fig. 9). Crosslinked gelatin (gel)-based graft copolymer nanoparticles were prepared using 2-hydroxyethyl methacrylate (HEMA) and/or styrene (Sty) monomers [113]. The prepared Gel/HEMA and Gel/Sty nanoparticles exhibited particle size ranging from 15 to 17 nm and from 0.42 to 5 μm, respectively with slow ibuprofen release within 6 h. Jătariu et al. [47] reported the preparation of nanoparticles based on gelatin and chitosan using a reverse emulsion-double crosslinking technique. Nanoparticles were formed by the dispersion of a solution of both polymers containing Tween 80 in toluene containing Span 80 to give a reverse W/O emulsion followed by double crosslinking. The nanoparticles proved to manifest pH sensitive interactions and thus can be considered good candidates for drug delivery.

Maillard reaction, a naturally occurring reaction that conjugates polysaccharide and protein by linking the reducing end carbonyl groups in the former to the amino groups in the latter, avoiding the use of toxic chemicals, was used to synthesize gelatin–dextran conjugate [42]. Afterward, complex coacervation core micelles (86 nm) were prepared *via* self-assembly of the gelatin–dextran conjugate with tea polyphenol (TPP) based on hydrophobic interaction and hydrogen bonding where the insoluble core of gelatin and TPP was stabilized by the conjugated hydrophilic dextran shell. Complex coacervation micelles offer several advantages including green process, narrow size distribution, high loading capacity, and sustained release.

8.2. Gelatin–lipid nanocomplexes

Gelatin–lipid hybrid nanoparticles (GLNPs) were prepared by a two-step desolvation method to improve the oral bioavailability of amphotericin B [109]. The drug was efficiently encapsulated within the lipid core, which was further stabilized by a biodegradable coat of gelatin, subsequently increasing the system's payload capability and providing additional protection in the gastrointestinal fluids. Electrostatic interaction between the cationic type A gelatin (below its pI) and the anionic lecithin was utilized for the formation of stable GLNPs. Moreover, hydrophobic interactions among the hydrophobic chains of lipid and hydrophobic residues of gelatin were also reported. GLNPs exhibited a sustained drug release profile, a 5.89- and 4.69-fold increase in the intestinal permeability and oral bioavailability, respectively, together with significantly lesser hemolytic and nephrotoxicity as compared to free drug. However, this system suffers from slight sensitivity to acidic degradation in the stomach; therefore, further attempts such as enteric coating of the formulation can be useful.

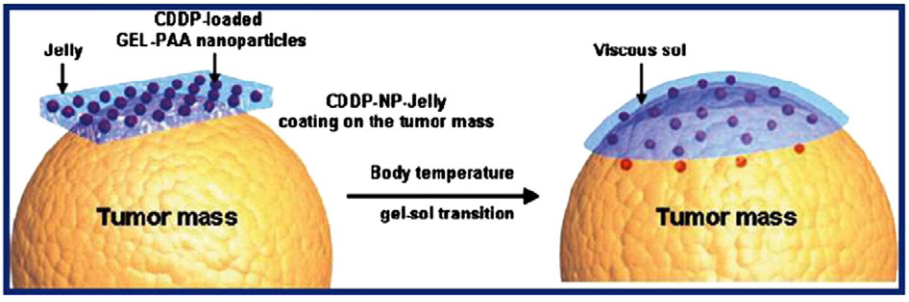

Fig. 9. Schematic illustration of CDDP-NP-Jelly coating on the tumor, which gradually transforms into a viscous sol *in vivo* due to the body temperature [74].

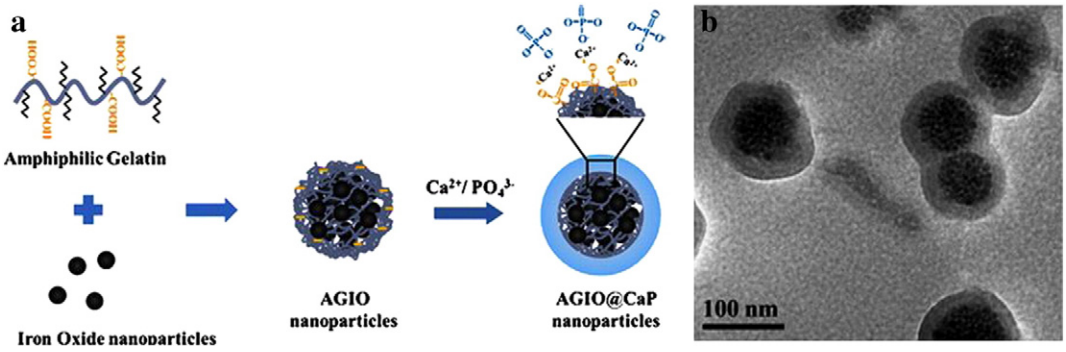

Fig. 10. (a) The synthetic scheme and (b) TEM image of AGIO nanoparticles with CaP coating. Modified from reference [68].

8.3. Gelatin–inorganic nanocomplexes

Gelatin was found to form nanocomplexes with various inorganic materials for drug delivery applications. Biocompatible hybrid gelatin–siloxane nanoparticles (GSNPs) were synthesized using a two-step sol–gel process using 3-glycidoxypropyl-trimethoxysilane and gelatin solution to yield a crosslinked network of gelatin–siloxane for drug delivery [140–142].

Multistage quantum dot GNPs (QDGNPs) composed of a gelatin core with amino-PEG QDs conjugated to the surface were successfully prepared [150]. After the original 100-nm GNPs preferentially extravasate from the leaky regions of the tumor vasculature into tumor tissue, a size change was triggered by proteases that are highly expressed in the tumor microenvironment such as matrix metalloproteinases, which degrade the cores of 100-nm GNPs, releasing smaller 10-nm nanoparticles (QDs) from their surface that can more readily diffuse throughout the tumor's interstitial space allowing penetration into the tumor parenchyma [150,151]. Ultimately, the smaller QDs will be replaced with a 10-nm nanocarrier of cancer therapeutics that are released as the particles penetrate deep into the tumor.

Magnetic gelatin nanocomplexes were elaborated by two methods: in the first one; iron oxide nanoparticles (IONPs) were encapsulated within gelatin through two-step desolvation method where gelatin was chemisorbed into IONP surface through the carbonyl and amino groups thus preventing the leaching of IONPs from nanoparticles. DOX [67] or cisplatin [75] were successfully loaded onto those nanoparticles with either adsorption or entrapment process. In the second method; amphiphilic hexanoyl-gelatin induced structural aggregation of IONPs to form an aggregated core on which positively charged Ca^{2+} ions (CaP) were adsorbed around the core by electrostatic interactions with the negatively charged carboxyl groups of amphiphilic gelatin to form core–shell nanoparticles [68]. DOX was encapsulated by electrolytic co-deposition during CaP shell formation (Fig. 10). After dissolution of the acid-sensitive CaP-DOX shell, DOX escaped from endosome and was delivered efficiently into the nucleus.

9. Drawbacks and challenges

Considerable attention has been directed to the application of GNPs. However, there are still critical problems associated with the use of animal-origin gelatins which carries with it the risk of contamination with transmissible spongiform encephalopathies (TSEs). In the case of gelatin however, the rigorous manufacturing processes such as acid, alkaline and heat treatments inactivate TSE agents and minimize TSE risk in drug products [26,41,56]. Furthermore, recombinant human gelatin (rHG) is commercially available. Currently, there are commercial suppliers (*e.g.* FibroGen South San Francisco, CA, USA) that produce gelatin by recombinant DNA technology (http://www.fibrogen.com). rHG is nontoxic and useful for developing nanostructures for drug delivery due to its nonimmunogenicity [26,41,56].

Commercial gelatins used in the pharmaceutical industry are heterogeneous mixtures of different sized proteins derived from bovine or porcine bones or skins with a wide range of molecular weights producing heterogeneous nanoparticle size distribution. An interesting strategy to overcome this drawback is the use of the two-step desolvation technique previously discussed [9] or the use of rHG due to its homogeneity in molecular weight and precisely defined properties to form nanoparticles with narrow size distribution. By overcoming these problems, the potential market for GNPs is expected to be huge.

References

[1] A. Kumari, S.K. Yadav, S.C. Yadav, Biodegradable polymeric nanoparticles based drug delivery systems, Colloids Surf. B: Biointerfaces 75 (2010) 1–18.
[2] A.O. Elzoghby, W.M. Samy, N.A. Elgindy, Protein-based nanocarriers as promising drug and gene delivery systems, J. Control. Release 161 (2012) 38–49.
[3] S. Kommareddy, D.B. Shenoy, M.M. Amiji, Gelatin nanoparticles and their biofunctionalization, in: C.S.S.R. Kumar (Ed.), Nanotechnologies for the Life Sciences, Vol. 1 Biofunctionalization of Nanomaterials, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2005, pp. 330–352.
[4] H. Wang, O.C. Boerman, K. Sariibrahimoglu, Y. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Comparison of micro- vs. nanostructured colloidal gelatin gels for sustained delivery of osteogenic proteins: bone morphogenetic protein-2 and alkaline phosphatase, Biomaterials 33 (2012) 8695–8703.
[5] S. Busch, U. Schwarz, R. Kniep, Chemical and structural investigations of biomimetically grown fluorapatite–gelatin composite aggregates, Adv. Funct. Mater. 13 (2003) 189–198.
[6] B. Mohanty, V.K. Aswal, J. Kohlbrecher, H.B. Bohidar, Synthesis of gelatin nanoparticles via simple coacervation, J. Surf. Sci. Technol. 21 (2005) 149–160.
[7] J.J. Marty, R.C. Oppenheim, P. Speiser, Nanoparticles—a new colloidal drug delivery system, Pharm. Acta Helv. 53 (1978) 17–23.
[8] C.A. Farrugia, M.J. Groves, Gelatin behaviour in dilute aqueous solution: designing a nanoparticulate formulation, J. Pharm. Pharmacol. 51 (1999) 643–649.
[9] C. Coester, K. Langer, H. Von Briesen, J. Kreuter, Gelatin nanoparticles by two-step desolvation—a new preparation method, surface modification and cell uptake, J. Microencapsul. 17 (2000) 187–193.
[10] K. Ofokansi, G. Winter, G. Fricker, C. Coester, Matrix-loaded biodegradable gelatin nanoparticles as new approach to improve drug loading and delivery, Eur. J. Pharm. Biopharm. 76 (2010) 1–9.
[11] Z. Lu, T.K. Yeh, M. Tsai, J.L.S. Au, M.G. Wientjes, Paclitaxel-loaded gelatin nanoparticles for intravesical bladder cancer therapy, Clin. Cancer Res. 10 (2004) 7677–7684.
[12] M.G. Cascone, L. Lazzeri, Z. Zho, Gelatin nanoparticles prepared by a simple W/O emulsion as delivery system for methotrexate, J. Mater. Sci. Mater. Med. 13 (2002) 523–526.
[13] A.K. Bajpai, J. Choubey, Design of gelatin nanoparticles as swelling controlled delivery system for chloroquine phosphate, J. Mater. Sci. Mater. Med. 17 (2006) 345–358.
[14] A.K. Bajpai, J. Choubey, In vitro release dynamics of an anticancer drug from swellable gelatin nanoparticles, J. Appl. Polym. Sci. 101 (2006) 2320–2332.
[15] J. Choubey, A.K. Bajpai, Investigation on magnetically controlled delivery of doxorubicin from superparamagnetic nanocarriers of gelatin crosslinked with genipin, J. Mater. Sci. Mater. Med. 21 (2010) 1573–1586.
[16] Y.-Z. Zhao, X. Li, C.-T. Lu, Y.-Y. Xu, H.-F. Lv, D.-D. Dai, L. Zhang, C.-Z. Sun, W. Yang, X.-K. Li, Y.-P. Zhao, H.-X. Fu, L. Cai, M. Lin, L.-J. Chen, M. Zhang, Experiment on the feasibility of using modified gelatin nanoparticles as insulin pulmonary administration system for diabetes therapy, Acta Diabetol. 49 (2012) 315–325.

[17] A.K. Gupta, M. Gupta, S.J. Yarwood, A.S.G. Curtis, Effect of cellular uptake of gelatin nanoparticles on adhesion, morphology and cytoskeleton organization of human fibroblasts, J. Control. Release 95 (2004) 197–207.
[18] M.J. Hou, M. Kim, D.O. Shah, A light scattering study on the droplet size and interdroplet interaction in microemulsion of AOT–oil water systems, J. Colloid Interface Sci. 123 (1998) 398–412.
[19] D.Q. Guerrero, E. Allemann, H. Fessi, E. Doelker, Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers, Drug Dev. Ind. Pharm. 24 (1998) 1113–1128.
[20] E.J. Lee, S.A. Khan, J.K. Park, K.-H. Lim, Studies on the characteristics of drug-loaded gelatin nanoparticles prepared by nanoprecipitation, Bioprocess Biosyst. Eng. 35 (2012) 297–307.
[21] U. Bilati, E. Allemann, E. Doelker, Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles, Eur. J. Pharm. Sci. 24 (2005) 67–75.
[22] S.A. Khan, M. Schneider, Improvement of nanoprecipitation technique for preparation of gelatin nanoparticles and potential macromolecular drug loading, Macromol. Biosci. 13 (2013) 455–463.
[23] S.Y. Fung, H. Yang, P. Chen, Formation of colloidal suspension of hydrophobic compounds with an amphiphilic self-assembling peptide, Colloids Surf. B: Biointerfaces 55 (2007) 200–211.
[24] K.J. Kim, Y. Byun, Preparation and characterizations of self-assembled PEGylated gelatin nanoparticles, Biotechnol. Bioprocess Eng. 4 (1999) 210–214.
[25] W.-M. Li, D.-M. Liu, S.-Y. Chen, Amphiphilically-modified gelatin nanoparticles: self-assembly behavior, controlled biodegradability, and rapid cellular uptake for intracellular drug delivery, J. Mater. Chem. 21 (2011) 12381–12388.
[26] Y.-W. Won, S.-M. Yoon, C.H. Sonn, K.-M. Lee, Y.-H. Kim, Nano self-assembly of recombinant human gelatin conjugated with α-tocopheryl succinate for Hsp90 inhibitor, 17-AAG, delivery, ACS Nano 5 (2011) 3839–3848.
[27] T. Tanigo, R. Takaoka, Y. Tabata, Sustained release of water-insoluble simvastatin from biodegradable hydrogel augments bone regeneration, J. Control. Release 143 (2010) 201–206.
[28] S. Han, M. Li, X. Liu, H. Gao, Y. Wu, Construction of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for doxorubicin delivery, Colloids Surf. B: Biointerfaces 102 (2013) 833–841.
[29] Y.-C. Chen, S.-H. Yu, G.-J. Tsai, D.-W. Tang, F.-L. Mi, Y.-P. Peng, Novel technology for the preparation of self-assembled catechin/gelatin nanoparticles and their characterization, J. Agric. Food Chem. 58 (2010) 6728–6734.
[30] Z. Li, L. Gu, Effects of mass ratio, pH, temperature, and reaction time on fabrication of partially purified pomegranate ellagitannin–gelatin nanoparticles, J. Agric. Food Chem. 59 (2011) 4225–4231.
[31] H. Ai, S.A. Jones, Y.M. Lvov, Biomedical application of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles, Cell Biochem. Biophys. 39 (2003) 23–43.
[32] T.G. Shutava, S.S. Balkundi, P. Vangala, J.J. Steffan, R.L. Bigelow, J.A. Cardelli, D.P. O'Neal, Y.M. Lvov, Layer-by-layer-coated gelatin nanoparticles as a vehicle for delivery of natural polyphenols, ACS Nano 3 (2009) 1877–1885.
[33] S. Kommareddy, M.M. Amiji, Preparation and loading of gelatin nanoparticles, Cold Spring Harb. Protoc. (2008), http://dx.doi.org/10.1101/pdb.prot4887.
[34] E. Leo, M.A. Vandelli, R. Cameroni, F. Forni, Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: involvement of the drug in the crosslinking process, Int. J. Pharm. 155 (1997) 75–82.
[35] M.J.A. Van Luyn, P.B. Van Wachem, P.J. Dijkstra, L.H.H. Olde Damink, J. Feijen, Calcification of subcutaneously implanted collagens in relation to cytotoxicity, cellular interactions and crosslinking, J. Mater. Sci. Mater. Med. 6 (1995) 288–296.
[36] B. Ballantyne, S.L. Jordan, Toxicological, medical, and industrial hygiene aspects of glutaraldehyde with particular reference to its biocidal use in cold sterilization procedures, J. Appl. Toxicol. 21 (2001) 131–151.
[37] M.A. Vandelli, F. Rivasi, P. Guerra, F. Forni, R. Arletti, Gelatin microspheres crosslinked with D,L-glyceraldehyde as a potential drug delivery system: preparation, characterisation, in vitro and in vivo studies, Int. J. Pharm. 215 (2001) 175–184.
[38] A.O. Elzoghby, W.M. Samy, N.A. Elgindy, Novel spray-dried genipin-crosslinked casein nanoparticles for prolonged release of alfuzosin hydrochloride, Pharm. Res. 30 (2013) 512–522.
[39] A.O. Elzoghby, M.W. Helmy, W.M. Samy, N.A. Elgindy, Spray-dried casein-based micelles as a vehicle for solubilization and controlled delivery of flutamide: formulation, characterization and in vivo pharmacokinetics, Eur. J. Pharm. Biopharm. 84 (2013) 487–496.
[40] F. Song, L.-M. Zhang, C. Yang, L. Yan, Genipin-crosslinked casein hydrogels for controlled drug delivery, Int. J. Pharm. 373 (2009) 41–47.
[41] Y.-W. Won, Y.-H. Kim, Recombinant human gelatin nanoparticles as a protein drug carrier, J. Control. Release 127 (2008) 154–161.
[42] H. Zhou, X. Sun, L. Zhang, P. Zhang, J. Li, Y.-N. Liu, Fabrication of biopolymeric complex coacervation core micelles for efficient tea polyphenol delivery via a green process, Langmuir 28 (2012) 14553–14561.
[43] N.T. Qazvini, S. Zinatloo, Synthesis and characterization of gelatin nanoparticles using CDI/NHS as a non-toxic crosslinking system, J. Mater. Sci. Mater. Med. 22 (2011) 63–69.
[44] R. Lantto, Protein crosslinking with oxidative enzymes and transglutaminase. Effects in meat protein systems, VTT Publ. 642 (2007) 1–114.
[45] T. Heidebach, P. Foerst, U. Kulozik, Transglutaminase-induced caseinate gelation for the microencapsulation of probiotic cells, Int. Dairy J. 19 (2008) 77–84.
[46] S. Fuchs, M. Kutscher, T. Hertel, G. Winter, M. Pietzsch, C. Coester, Transglutaminase: new insights into gelatin nanoparticle crosslinking, J. Microencapsul. 27 (2010) 747–754.
[47] A.N. Jătariu, M.N. Holban, C.A. Peptu, A. Sava, M. Costuleanu, M. Popa, Double crosslinked interpenetrated network in nanoparticle form for drug targeting—preparation, characterization and biodistribution studies, Int. J. Pharm. 436 (2012) 66–74.
[48] D. Narayanan, M.G. Geena, H. Lakshmi, M. Koyakutty, S. Nair, D. Menon, Poly-(ethylene glycol) modified gelatin nanoparticles for sustained delivery of the anti-inflammatory drug ibuprofen-sodium: an in vitro and in vivo analysis, Nanomedicine 9 (2013) 818–828.
[49] M. Nahar, D. Mishra, V. Dubey, N.K. Jain, Development, characterization, and toxicity evaluation of amphotericin B–loaded gelatin nanoparticles, Nanomedicine 4 (2008) 252–261.
[50] B. Zhao, J. Xie, J. Zhao, A novel water-soluble nanoparticles of hypocrellin B and their interaction with a model protein: C-phycocyanin, Biochim. Biophys. Acta 1670 (2004) 113–120.
[51] J. Vandervoort, A. Ludwig, Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use, Eur. J. Pharm. Biopharm. 57 (2004) 251–261.
[52] V.L. Truong-Le, J.T. August, K.W. Leong, Controlled gene delivery by DNA–gelatin nanospheres, Hum. Gene Ther. 9 (1998) 1709–1717.
[53] A. Saxena, K. Sachin, H.B. Bohidar, A.K. Verma, Effect of molecular weight heterogeneity on drug encapsulation efficiency of gelatin nanoparticles, Colloids Surf. B: Biointerfaces 45 (2005) 42–48.
[54] G. Kaul, M. Amiji, Long-circulating poly(ethylene glycol)-modified gelatin nanoparticles for intracellular delivery, Pharm. Res. 19 (2002) 1062–1068.
[55] E. Leo, R. Cameroni, F. Forni, Dynamic dialysis for the drug release evaluation from doxorubicin–gelatin nanoparticle conjugates, Int. J. Pharm. 180 (1999) 23–30.
[56] Y.-W. Won, Y.-H. Kim, Preparation and cytotoxicity comparison of type A gelatin nanoparticles with recombinant human gelatin nanoparticles, Macromol. Res. 17 (2009) 464–468.
[57] S. Azarmi, Y. Huang, H. Chen, S. McQuarrie, D. Abrams, W. Roa, W.H. Finlay, G.G. Miller, R. Löbenberg, Optimization of a two-step desolvation method for preparing gelatin nanoparticles and cell uptake studies in 143B osteosarcoma cancer cells, J. Pharm. Pharm. Sci. 9 (2006) 124–132.
[58] M. Brzoska, K. Langer, C. Coester, S. Loitsch, T.O.F. Wagner, C.v. Mallinckrodt, Incorporation of biodegradable nanoparticles into human airway epithelium cells—in vitro study of the suitability as a vehicle for drug or gene delivery in pulmonary diseases, Biochem. Biophys. Res. Commun. 318 (2004) 562–570.
[59] C. Coester, P. Nayyar, J. Samuel, In vitro uptake of gelatin nanoparticles by murine dendritic cells and their intracellular localization, Eur. J. Pharm. Biopharm. 62 (2006) 306–314.
[60] S. Karthikeyan, N.R. Prasad, A. Ganamani, E. Balamurugan, Anticancer activity of resveratrol-loaded gelatin nanoparticles on NCI-H460 non-small cell lung cancer cells, Biomed. Prev. Nutr. 3 (2013) 64–73.
[61] N. Kuntworbe, R. Al-Kassas, Design and in vitro haemolytic evaluation of cryptolepine hydrochloride-loaded gelatin nanoparticles as a novel approach for the treatment of malaria, AAPS PharmSciTech 13 (2012) 568–581.
[62] J.C. Zillies, K. Zwiorek, F. Hoffmann, A. Vollmar, T.J. Anchordoquy, G. Winter, C. Coester, Formulation development of freeze-dried oligonucleotide-loaded gelatin nanoparticles, Eur. J. Pharm. Biopharm. 70 (2008) 514–521.
[63] E. Leo, R. Arletti, F. Forni, R. Cameroni, General and cardiac toxicity of doxorubicin-loaded gelatin nanoparticles, Farmaco 52 (1997) 385–388.
[64] G.Y. Lee, K. Park, J.H. Nam, S.Y. Kim, Y. Byun, Anti-tumor and anti-metastatic effects of gelatin–doxorubicin and PEGylated gelatin–doxorubicin nanoparticles in SCC7 bearing mice, J. Drug Target. 14 (2006) 707–716.
[65] P. Dwivedi, S. Kansal, M. Sharma, R. Shukla, A. Verma, P. Shukla, P. Tripathi, P. Gupta, D. Saini, K. Khandelwal, R. Verma, A.K. Dwivedi, P.R. Mishra, Exploiting 4-sulphate N-acetyl galactosamine decorated gelatin nanoparticles for effective targeting to professional phagocytes in vitro and in vivo, J. Drug Target. 20 (2012) 883–896.
[66] M. Garg, J. Madan, R.S. Pandey, S. Sardana, A. Katyal, R. Chandra, Galactosylated gelatin nanovectors of doxorubicin inhibit cell proliferation and induce apoptosis in hepatocarcinoma cells, Anticancer Drugs 23 (2012) 836–845.
[67] B. Gaihre, M.S. Khil, D.R. Lee, H.Y. Kim, Gelatin-coated magnetic iron oxide nanoparticles as carrier system: drug loading and in vitro drug release study, Int. J. Pharm. 365 (2009) 180–189.
[68] W.-M. Li, S.-Y. Chen, D.-M. Liu, In situ doxorubicin–CaP shell formation on amphiphilic gelatin–iron oxide core as a multifunctional drug delivery system with improved cytocompatibility, pH-responsive drug release and MR imaging, Acta Biomater. 9 (2013) 5360–5368.
[69] T.K. Yeh, Z. Lu, M.G. Wientjes, J.L.-S. Au, Formulating paclitaxel in nanoparticles alters its disposition, Pharm. Res. 22 (2005) 867–874.
[70] C.-L. Tseng, W.-Y. Su, K.-C. Yen, K.-C. Yang, F.-H. Lin, The use of biotinylated-EGF-modified gelatin nanoparticle carrier to enhance cisplatin accumulation in cancerous lungs via inhalation, Biomaterials 30 (2009) 3476–3485.
[71] A. Jain, A. Gulbake, A. Jain, S. Shilpi, P. Hurkat, A. Jain, S.K. Jain, Development of surface-functionalised nanoparticles for FGF2 receptor-based solid tumour targeting, J. Microencapsul. 29 (2012) 95–102.
[72] D. Ding, Z. Zhu, Q. Liu, J. Wang, Y. Hua, X. Jiang, B. Liu, Cisplatin-loaded gelatin-poly(acrylic acid) nanoparticles: synthesis, antitumor efficiency in vivo and penetration in tumors, Eur. J. Pharm. Biopharm. 79 (2011) 142–149.
[73] D. Ding, J. Wang, Z. Zhu, R. Li, W. Wu, B. Liu, X. Jiang, Tumor accumulation, penetration, and antitumor response of cisplatin-loaded gelatin/poly(acrylic acid) nanoparticles, ACS Appl. Mater. Interfaces 4 (2012) 1838–1846.
[74] D. Ding, Z.S. Zhu, R.T. Li, X.L. Li, W. Wu, X.Q. Jiang, B.R. Liu, Nanospheres-incorporated implantable hydrogel as a trans-tissue drug delivery system, ACS Nano 5 (2011) 2520–2534.

[75] H. Yılmaz, S.H. Sanlıer, Preparation of magnetic gelatin nanoparticles and investigating the possible use as chemotherapeutic agent, Artif. Cells Nanomed. Biotechnol. 41 (2013) 69–77.
[76] J. Madan, N. Dhiman, S. Sardana, R. Aneja, R. Chandra, A. Katyal, Long-circulating poly(ethylene glycol)-grafted gelatin nanoparticles customized for intracellular delivery of noscapine: preparation, in-vitro characterization, structure elucidation, pharmacokinetics, and cytotoxicity analyses, Anticancer Drugs 22 (2011) 543–555.
[77] J.K. Li, N. Wang, X.S. Wu, A novel biodegradable system based on gelatin nanoparticles and poly(lactic-coglycolic acid) microspheres for protein and peptide drug delivery, J. Pharm. Sci. 86 (1997) 891–895.
[78] J.K. Li, N. Wang, X.S. Wu, Gelatin nanoencapsulation of protein/peptide drugs using an emulsifier-free emulsion method, J. Microencapsul. 15 (1998) 163–172.
[79] Y. Uesugi, H. Kawata, J.-i. Jo, Y. Saito, Y. Tabata, An ultrasound-responsive nano delivery system of tissue-type plasminogen activator for thrombolytic therapy, J. Control. Release 147 (2010) 269–277.
[80] Y. Uesugi, H. Kawata, Y. Saito, Y. Tabata, Ultrasound-responsive thrombus treatment with zinc-stabilized gelatin nano-complexes of tissue-type plasminogen activator, J. Drug Target. 20 (2012) 224–234.
[81] H. Wang, Q. Zou, O.C. Boerman, A.W.G. Nijhuis, J.A. Jansen, Y. Li, S.C.G. Leeuwenburgh, Combined delivery of BMP-2 and bFGF from nanostructured colloidal gelatin gels and its effect on bone regeneration in vivo, J. Control. Release 166 (2013) 172–181.
[82] M.S. Sudheesh, S.P. Vyas, D.V. Kohli, Nanoparticle-based immunopotentiation via tetanus toxoid loaded gelatin and aminated gelatin nanoparticles, Drug Deliv. 18 (2011) 320–330.
[83] G. Kaul, M. Amiji, Protein nanoparticles for gene delivery, in: M. Amiji (Ed.), Polymeric Gene Delivery: Principles and Applications, CRC Press LLC, Boca Raton, FL, 2005, pp. 429–447.
[84] P. Magadala, M. Amiji, Epidermal growth factor receptor-targeted gelatin-based engineered nanocarriers for DNA delivery and transfection in human pancreatic cancer cells, AAPS J. 10 (2008) 565–576.
[85] K. Zwiorek, C. Bourquin, J. Battiany, G. Winter, S. Endres, G. Hartmann, C. Coester, Delivery by cationic gelatin nanoparticles strongly increases the immunostimulatory effects of CpG oligonucleotides, Pharm. Res. 25 (2008) 551–562.
[86] C. Coester, J. Kreuter, H. von Briesen, K. Langer, Preparation of avidin-labelled gelatin nanoparticles as carriers for biotinylated peptide nucleic acid, Int. J. Pharm. 196 (2000) 147–149.
[87] G. Kaul, C. Lee-Parsons, M. Amiji, Poly(ethylene glycol)-modified gelatin nanoparticles for intracellular delivery, Pharm. Eng. 23 (2003) 1–5.
[88] G. Kaul, M. Amiji, Cellular interactions and in vitro DNA transfection studies with poly(ethylene glycol)-modified gelatin nanoparticles, J. Pharm. Sci. 94 (2005) 184–198.
[89] K.W. Leong, H.Q. Mao, V.L. Truong-Le, K. Roy, S.M. Walsh, J.T. August, DNA-polycation nanospheres as non-viral gene delivery vehicles, J. Control. Release 53 (1998) 183–193.
[90] J. Xu, S. Ganesh, M. Amiji, Non-condensing polymeric nanoparticles for targeted gene and siRNA delivery, Int. J. Pharm. 427 (2012) 21–34.
[91] S. Shah, Hypoxia in Tumor Angiogenesis and Metastasis: Evaluation of VEGF and MMP Over-Expression and Down-Regulation of HIF-1α with RNAi in Hypoxic Tumor Cells, 2010. (Master's Thesis).
[92] M.D. Bhavsar, S.B. Tiwari, M.M. Amiji, Formulation optimization for the nanoparticles-in-microsphere hybrid oral delivery system using factorial design, J. Control. Release 110 (2006) 422–430.
[93] M.D. Bhavsar, M.M. Amiji, Gastrointestinal distribution and in vivo gene transfection studies with nanoparticles-in-microsphere oral system (NiMOS), J. Control. Release 119 (2007) 339–348.
[94] M.D. Bhavsar, M.M. Amiji, Development of novel biodegradable polymeric nanoparticles-in-microsphere formulation for local plasmid DNA delivery in the gastrointestinal tract, AAPS PharmSciTech 9 (2008) 288–294.
[95] C. Kriegel, H. Attarwala, M. Amiji, Multi-compartmental oral delivery systems for nucleic acid therapy in the gastrointestinal tract, Adv. Drug Deliv. Rev. 65 (2013) 891–901.
[96] M.D. Bhavsar, M.M. Amiji, Oral IL-10 gene delivery in a microsphere-based formulation for local transfection and therapeutic efficacy in inflammatory bowel disease, Gene Ther. 15 (2008) 1200–1209.
[97] C. Kriegel, M. Amiji, Oral TNF-α gene silencing using a polymeric microsphere-based delivery system for the treatment of inflammatory bowel disease, J. Control. Release 150 (2011) 77–86.
[98] W. Friess, Collagen-material for drug delivery, Eur. J. Pharm. Biopharm. 45 (1998) 113–136.
[99] G.K. Zorzi, J.E. Párraga, B. Seijo, A. Sánchez, Hybrid nanoparticle design based on cationized gelatin and the polyanions dextran sulfate and chondroitin sulfate for ocular gene therapy, Macromol. Biosci. 11 (2011) 905–913.
[100] G.K. Zorzi, L. Contreras-Ruiz, J.E. Párraga, A. López-García, R.R. Bello, Y. Diebold, B. Seijo, A. Sánchez, Expression of MUC5AC in ocular surface epithelial cells using cationized gelatin nanoparticles, Mol. Pharm. 8 (2011) 1783–1788.
[101] J.O.-H. Sham, Y. Zhang, W.H. Finlay, W.H. Roa, R. Löbenberg, Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to the lung, Int. J. Pharm. 269 (2004) 457–467.
[102] S. Fuchs, J. Klier, A. May, G. Winter, C. Coester, H. Gehlen, Towards an inhalative in vivo application of immunomodulating gelatin nanoparticles in horse-related preformulation studies, J. Microencapsul. 29 (2012) 615–625.
[103] J. Klier, S. Fuchs, A. May, U. Schillinger, C. Plank, G. Winter, H. Gehlen, C. Coester, A nebulized gelatin nanoparticle-based CpG formulation is effective in immunotherapy of allergic horses, Pharm. Res. 29 (2012) 1650–1657.
[104] T. Zou, S.S. Percival, Q. Cheng, Z. Li, C.A. Rowe, L. Gu, Preparation, characterization, and induction of cell apoptosis of cocoa procyanidins–gelatin–chitosan nanoparticles, Eur. J. Pharm. Biopharm. 82 (2012) 36–42.
[105] Z. Gan, T. Zhang, Y. Liu, D. Wu, Temperature-triggered enzyme immobilization and release based on cross-linked gelatin nanoparticles, PLoS One 7 (2012) e47154.
[106] S.K. Jain, Y. Gupta, A. Jain, A.R. Saxena, P. Khare, A. Jain, Mannosylated gelatin nanoparticles bearing an anti-HIV drug didanosine for site-specific delivery, Nanomedicine 4 (2008) 41–48.
[107] N. Kuntworbe, M. Ofori, P. Addo, M. Tingle, R. Al-Kassas, Pharmacokinetics and in vivo chemosuppressive activity studies on cryptolepine hydrochloride and cryptolepine hydrochloride-loaded gelatin nanoformulation designed for parenteral administration for the treatment of malaria, Acta Trop. 127 (2013) 165–173.
[108] M. Nahar, V. Dubey, D. Mishra, P.K. Mishra, A. Dube, N.K. Jain, In vitro evaluation of surface functionalized gelatin nanoparticles for macrophage targeting in the therapy of visceral leishmaniasis, J. Drug Target. 18 (2010) 93–105.
[109] S. Jain, P.U. Valvi, N.K. Swarnakar, K. Thanki, Gelatin coated hybrid lipid nanoparticles for oral delivery of amphotericin B, Mol. Pharm. 9 (2012) 2542–2553.
[110] G.K. Saraogi, P. Gupta, U.D. Gupta, N.K. Jain, G.P. Agrawal, Gelatin nanocarriers as potential vectors for effective management of tuberculosis, Int. J. Pharm. 385 (2010) 143–149.
[111] G.K. Saraogi, B. Sharma, B. Joshi, P. Gupta, U.D. Gupta, N.K. Jain, G.P. Agrawal, Mannosylated gelatin nanoparticles bearing isoniazid for effective management of tuberculosis, J. Drug Target. 19 (2011) 219–227.
[112] D. Jain, R. Banerjee, Comparison of ciprofloxacin hydrochloride-loaded protein, lipid, and chitosan nanoparticles for drug delivery, J. Biomed. Mater. Res. B Appl. Biomater. 86 (2008) 105–112.
[113] A.A. Haroun, N.R. El-Halawany, C. Loira-Pastoriza, P. Maincent, Synthesis and in vitro release study of ibuprofen-loaded gelatin graft copolymer nanoparticles, Drug Dev. Ind. Pharm. (2013), http://dx.doi.org/10.3109/03639045.2012.746359.
[114] R. Kumar, R.C. Nagarwal, M. Dhanawat, J.K. Pandit, In-vitro and in-vivo study of indomethacin loaded gelatin nanoparticles, J. Biomed. Nanotechnol. 7 (2011) 325–333.
[115] V. Singh, A.K. Chaudhary, Development and characterization of rosiglitazone loaded gelatin nanoparticles using two step desolvation method, Int. J. Pharm. Sci. Rev. Res. 5 (2010) 100–103.
[116] S. Kommareddy, M. Amiji, Preparation and evaluation of thiol-modified gelatin nanoparticles for intracellular DNA delivery in response to glutathione, Bioconjug. Chem. 16 (2005) 1423–1432.
[117] S. Kommareddy, M. Amiji, Poly(ethylene glycol)-modified thiolated gelatin nanoparticles for glutathione-responsive intracellular DNA delivery, Nanomedicine 3 (2007) 32–42.
[118] H. Otsuka, Y. Nagasaki, K. Kataoka, PEGylated nanoparticles for biological and pharmaceutical applications, Adv. Drug Deliv. Rev. 55 (2003) 403–419.
[119] J. Crawford, Clinical uses of pegylated pharmaceuticals in oncology, Cancer Treat. Rev. 28 (2002) 7–11.
[120] G. Kaul, M. Amiji, Biodistribution and targeting potential of poly(ethylene glycol)-modified gelatin nanoparticles in subcutaneous murine tumor model, J. Drug Target. 12 (2004) 585–591.
[121] G. Kaul, M. Amiji, Tumor-targeted gene delivery using poly(ethylene glycol)-modified gelatin nanoparticles: in vitro and in vivo studies, Pharm. Res. 22 (2005) 951–961.
[122] T. Kushibiki, H. Matsuoka, Y. Tabata, Synthesis and physical characterization of poly(ethylene glycol)-gelatin conjugates, Biomacromolecules 5 (2004) 202–208.
[123] S. Kommareddy, M. Amiji, Biodistribution and pharmacokinetic analysis of long-circulating thiolated gelatin nanoparticles following systemic administration in breast cancer-bearing mice, J. Pharm. Sci. 96 (2007) 397–407.
[124] A. Babu, J. Periasamy, A. Gunasekaran, G. Kumaresan, S. Naicker, P. Gunasekaran, R. Murugesan, Polyethylene glycol-modified gelatin/polylactic acid nanoparticles for enhanced photodynamic efficacy of a hypocrellin derivative in vitro, J. Biomed. Nanotechnol. 9 (2013) 177–192.
[125] K. Zwiorek, J. Kloeckner, E. Wagner, C. Coester, Gelatin nanoparticles as a new and simple gene delivery system, J. Pharm. Pharm. Sci. 7 (2005) 22–28.
[126] X. Xu, R.M. Capito, M. Spector, Delivery of plasmid IGF-1 to chondrocytes via cationized gelatin nanoparticles, J. Biomed. Mater. Res. 84A (2008) 73–83.
[127] H. Ishikawa, Y. Nakamura, J.-i. Jo, Y. Tabata, Gelatin nanospheres incorporating siRNA for controlled intracellular release, Biomaterials 33 (2012) 9097–9104.
[128] W.-T. Kuo, H.-Y. Huang, M.-J. Chou, M.-C. Wu, Y.-Y. Huang, Surface modification of gelatin nanoparticles with polyethylenimine as gene vector, J. Nanomater. 2011 (2011) 1–5.
[129] H. Mimi, K.M. Ho, Y.S. Siu, A. Wu, P. Li, Polyethyleneimine-based core–shell nanogels: a promising siRNA carrier for argininosuccinate synthetase mRNA knockdown in HeLa cells, J. Control. Release 158 (2012) 123–130.
[130] L. Rabinovick-Guilatt, P. Couvreur, G. Lambert, C. Dubernet, Cationic vectors in ocular drug delivery, J. Drug Target. 12 (2004) 623–633.
[131] J.A. Weigel, P.H. Weigel, Characterization of the recombinant rat 175-kDa hyaluronan receptor for endocytosis (HARE), J. Biol. Chem. 278 (2003) 42802–42811.
[132] S. Balthasar, K. Michaelis, N. Dinauer, H. von Briesen, J. Kreuter, K. Langer, Preparation and characterization of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes, Biomaterials 26 (2005) 2723–2732.
[133] N. Dinauer, S. Balthasar, C. Weber, J. Kreuter, K. Langer, H. von Briesen, Selective targeting of antibody-conjugated nanoparticles to leukemic cells and primary T-lymphocytes, Biomaterials 26 (2005) 5898–5906.
[134] Z. Li, R. Zhao, X. Wu, Y. Sun, M. Yao, J. Li, Y. Xu, J. Gu, Identification and characterization of a novel peptide ligand of epidermal growth factor receptor for targeted delivery of therapeutics, FASEB J. 19 (2005) 1978–1985.

[135] J. Xu, M. Amiji, Therapeutic gene delivery and transfection in human pancreatic cancer cells using epidermal growth factor receptor targeted gelatin nanoparticles, J. Vis. Exp. 59 (2012) e3612.
[136] J. Xu, F. Gattacceca, M. Amiji, Biodistribution and pharmacokinetics of EGFR-targeted thiolated gelatin nanoparticles following systemic administration in pancreatic tumor-bearing mice, Mol. Pharm. 10 (2013) 2031–2044.
[137] C.-L. Tseng, S.Y.-H. Wu, W.-H. Wang, C.-L. Peng, F.-H. Lin, C.-C. Lin, T.-H. Young, M.-J. Shieh, Targeting efficiency and biodistribution of biotinylated-EGF-conjugated gelatin nanoparticles administered via aerosol delivery in nude mice with lung cancer, Biomaterials 29 (2008) 3014–3022.
[138] G. Drin, S. Cottin, E. Blanc, A.R. Rees, J. Temsamani, Studies on the internalization mechanism of cationic cell-penetrating peptides, J. Biol. Chem. 278 (2003) 31192–31201.
[139] B. Wei, Y. Wei, K. Zhang, J. Wang, R. Xu, S. Zhan, G. Lin, W. Wang, M. Liu, L. Wang, R. Zhang, J. Li, Development of an antisense RNA delivery system using conjugates of the MS2 bacteriophage capsids and HIV-1 TAT cell-penetrating peptide, Biomed. Pharmacother. 63 (2009) 313–318.
[140] X.-h. Tian, F. Wei, T.-x. Wang, D. Wang, J. Wang, X.-n. Lin, P. Wang, L. Ren, Blood–brain barrier transport of Tat peptide and polyethylene glycol decorated gelatin–siloxane nanoparticle, Mater. Lett. 68 (2012) 94–96.
[141] X.-h. Tian, F. Wei, T.-x. Wang, P. Wang, X.-n. Lin, J. Wang, D. Wang, L. Ren, In vitro and in vivo studies on gelatin–siloxane nanoparticles conjugated with SynB peptide to increase drug delivery to the brain, Int. J. Nanomedicine 7 (2012) 1031–1041.
[142] X.-H. Tian, Z.-G. Wang, H. Meng, Y.-H. Wang, W. Feng, F. Wei, Z.-C. Huang, X.-N. Lin, L. Ren, Tat peptide-decorated gelatin–siloxane nanoparticles for delivery of CGRP transgene in treatment of cerebral vasospasm, Int. J. Nanomedicine 8 (2013) 865–876.
[143] N. Nimje, A. Agarwal, G.K. Saraogi, N. Lariya, G. Rai, H. Agrawal, G.P. Agrawal, Mannosylated nanoparticulate carriers of rifabutin for alveolar targeting, J. Drug Target. 17 (2009) 777–787.
[144] B. Lepenies, J. Yin, P.H. Seeberger, Applications of synthetic carbohydrates to chemical biology, Curr. Opin. Chem. Biol. 14 (2010) 404–411.
[145] R.T. Lee, M.H. Wang, W.J. Lin, Y.C. Lee, New and more efficient multivalent glyco-ligands for asialoglycoprotein receptor of mammalian hepatocytes, Bioorg. Med. Chem. 19 (2011) 2494–2500.
[146] S. Elbert, J.A. Hubbell, Development of fibrin derivatives for controlled release of heparin-binding growth factors, J. Control. Release 65 (2000) 389–402.
[147] D. Sarkar, Fabrication of an optimized fluorescer encapsulated polymer coated gelatin nanoparticle and study of its retarded release properties, J. Photochem. Photobiol. A 252 (2013) 194–202.
[148] H. Wang, M.B. Hansen, D.W.P.M. Löwik, J.C.M. van Hest, Y. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Oppositely charged gelatin nanospheres as building blocks for injectable and biodegradable gels, Adv. Mater. 23 (2011) H119–H124.
[149] Y. Wang, Y. Zhang, W. Du, C. Wu, J. Zhao, Intelligent core–shell nanoparticles and hollow spheres based on gelatin and PAA via template polymerization, J. Colloid Interface Sci. 334 (2009) 153–160.
[150] C. Wong, T. Stylianopoulos, J. Cui, J. Martin, V.P. Chauhan, W. Jiang, Z. Popović, R.K. Jain, M.G. Bawendi, D. Fukumura, Multistage nanoparticle delivery system for deep penetration into tumor tissue, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 2426–2431.
[151] E.I. Deryugina, M.A. Bourdon, R.A. Reisfeld, A. Strongin, Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2, Cancer Res. 58 (1998) 3743–3750.