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REVIEW

Recent advances in the use of gelatin in biomedical research

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Abstract The biomacromolecule, gelatin, has increasingly been used in biomedicine—beyond its traditional use in food and cosmetics. The appealing advantages of gelatin, such as its cell-adhesive structure, low cost, off-the-shelf availability, high biocompatibility, biodegradability and low immunogenicity, among others, have made it a desirable candidate for the development of biomaterials for tissue engineering and drug delivery. Gelatin can be formulated in the form of nanoparticles, employed as size-controllable porogen, adopted as surface coating agent and mixed with synthetic or natural biopolymers forming composite scaffolds. In this article, we review recent advances in the versatile applications of gelatin within biomedical context and attempt to draw upon its advantages and potential challenges.

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Introduction

The demand for bioactive molecules with reliable properties, versatile functions and comparatively low cost has fuelled the research and development of functional proteins. One of the most notable candidates is gelatin, a denatured protein obtained by hydrolysis of animal collagen with either acid or alkaline. Gelatin has a long history of safe use in pharmaceuticals, cosmetics and food products, and is considered as a generally-regarded-as-safe (GRAS) material by the United States Food and Drug Administration (FDA) (Elzoghby et al. 2012). In the clinic, gelatin has been used as a plasma expander or in the form of gelatin sponge (Gelfoam), as well as being included in vaccines and other protein formulations.

Gelatin has broad advantages for biomedical pharmaceutical applications. Firstly, it is cheap and readily available, with high biocompatibility and biodegradability. Secondly, as a denatured product, gelatin is much less antigenic than collagen (Elzoghby et al. 2012). Thirdly, the gelatin chains contain abundant motifs such as arginine-glycine-aspartic (RGD) sequences that modulate cell adhesion, thereby improving the final biological behaviour over polymers that lack these cell-recognition sites (Wang et al.

2012). Fourthly, its diverse and accessible functional groups allow for chemical modifications, such as coupling with crosslinkers and targeting-ligands, which further enable the development of targeted drug delivery vehicles (Ayre et al. 2014). Lastly, gelatin has versatile functions in tissue engineering applications—as a matrix, it supports therapeutic cell adhesion without comprising cell phenotypes (Wang et al. 2012) (Cheng et al. 2012; Ruggeri et al. 2014); whilst as a porogenic agent, it provides intermediate structural support when blending with other material constituents (Tang et al. 2012b).

All these advantages of gelatin prepare it a promising natural macromolecule for drug delivery, tissue engineering and cell therapy. Mostly based on literatures within the past 5 years, this review aims to first introduce the appealing, tuneable characteristics of gelatin, and next discuss its particular applications in drug/cell delivery and tissue engineering, before finally addressing some emerging concerns over the use of gelatin and proposing an insight of the possible challenges ahead.

Gelatin characterisation

Gelatin has a unique sequence of amino acids. It is obtained through hydrolysis of collagen, which is the major component of the connective tissues like skin, tendon and bone, among others. Collagen contains interconnected protein chains. Hydrolysis breaks up the tertiary structure of collagen and produces gelatin, both sharing a primary structure with as many as 20 different amino acids in variable proportions. In gelatin, the amino acid composition and its sequence differs from one source to another, though always comprising non-polar and polar amino acids. For example, amino acids in pigskin gelatin and bone gelatin do not contain cysteine, which is present in fish scale and bone, having on contrary lower content of glycine (Gly) in comparison with mammalian sources (Zhang et al. 2011). All gelatins, except for pigskin gelatin, do not contain aspartic acid and glutamic acid. Structurally, collagen and gelatin molecules contain repeating sequences of Gly-X–Y triplets, where X and Y are mostly proline (Pro) and hydroxyproline (Hypro) amino acids (Duan et al. 2011). Gly, being dominant component, is the smallest amino acid as its lateral group is a hydrogen. Pro and Hypro—with rigid lateral pyrrolidine rings—display steric hindrances. Moreover, chemical composition and distribution of particular amino acids may affect rigidity of gelatin chain.

Each amino acid chain may have molecular weight between 10,000 and several hundred thousands of Daltons, depending upon the raw material and conditions of the conversion of collagen into gelatin. During the extraction process, tropocollagen molecules, made of three polypeptide chains arranged in triple helices, are broken down into single chains (called α chains with molecular weight around 100,000), covalently linked double α-chain species (β chains), and triple α-chain species (γ chains) or lower polypeptides. The extraction and pre-extraction parameters, like pH, temperature, and extraction time, have an impact on the amount of each chains and therefore determine the resulting features of gelatin, including molecular weight (Xu et al. 2014). Two types of gelatin are generally obtainable, depending on the pre-treatment procedure (prior to extraction process). Acidic pre-treatment (type A) barely affects the amide groups while the alkaline pre-treatment (type B) targets the amide groups of asparagines and glutamine and hydrolyzes them into carboxyl groups, thus converting many of these residues to aspartate and glutamate. As a protein, gelatin exhibits an amphoteric behaviour due to the presence of both acidic and basic functional groups, as a result of existence of amino acids functional groups and terminal amino and carboxyl groups.

Gelatin as tissue engineering scaffolds

Particularly attractive in tissue engineering are nano- and submicron-sized fibres formed from natural polymers (biopolymers) as scaffolds, which have potential to substitute native extracellular matrices (ECM). One of the most promising biopolymers for formation of scaffolds is gelatin (Jiang et al. 2014). Despite the fact that gelatin is obtained from native collagen via hydrolysis, leading to separation of molecules from original α-helix conformation by breaking of intermolecular bonds, individual molecules of gelatin still maintain their primary structure. This primary structure provides the RGD amino acid sequence, which is recognition sequence for integrin-mediated cell adhesion. According to the literature, gelatin used as scaffold component improves significantly infiltration, adhesion, spreading, and

proliferation of cells on resulting scaffolds. Introduction of natural biopolymers has beneficial effect on biological recognition signals and thus cells are expected to migrate deeper into the scaffold. Additionally, improved elasticity and deformability facilitate formation of new or expansion of existing cavities for cell penetration.

Gelatin mixed with ceramics

The skeleton is a composite system, consisting of protein polymers and ceramics. Attempts have been made to create similar composites with gelatin and ceramics, in efforts to promote bone regeneration. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$(HA), is a naturally-occurring mineral which constitutes the inorganic component of bone matrix. As an example of a simple two-phase composite, porous HA can be coated with gelatin in order to increase construct mechanical properties. This increase was shown to be proportional to the concentration of gelatin, leading to the hypothesis that gelatin increases HA toughness by bridging material cracks (Dressler et al. 2011).

Using a similar HA precipitation-based strategy, porous HA-gelatin microparticles were fabricated with fairly uniform size distribution in a one pot reaction using vegetable oil rather than harsh organic solvents (Ródenas-Rochina et al. 2013). Alternatively, HA-gelatin microparticles were synthesised by the addition of granulated coralline HA to a solution of gelatin, followed by dropwise addition of the mixture to a polymethylmethacrylate (PMMA) dispersion and glutaraldehyde cross-linking (Farbod et al. 2014). This second method of HA-gelatin composite microparticle synthesis resulted in more variable microparticle size, with diameters ranging from 1 to 100 μm (mean size ~16 μm). Compared with HA microparticles without gelatin, cells seeded on composite microparticles accelerated the proliferation of human osteoblast-like Saos-2 cells, suggesting that this gelatin-HA system could potentially be used as an injectable carrier for cell delivery (Perez et al. 2011).

Gelatin integrated into naturally-derived polymers

Naturally-occurring polymers such as collagen are the building blocks of the extracellular matrix and are therefore attractive materials for tissue engineering. These materials include polymers synthesised by human cells (such as collagen and hyaluronan) as well as naturally-derived polymers synthesized by other organisms (such as chitin and silk) (Wan and Tai 2013; Kundu et al. 2014). Composites of gelatin and other naturally-occurring polymers offer good biocompatibility and enable biomimetic strategies.

Hyaluronan

Hyaluronan is a ubiquitous polysaccharide with a high density of negative charge (allowing for water retention) and the ability to offer binding sites for molecules (Lam et al. 2014). However, the hydrophilicity of hyaluronan can inhibit protein adsorption and consequently prevent cell attachment. Therefore, gelatin-hyaluronan composites have been explored in order to utilise the advantages of both materials. One strategy is to add thiol functional groups to both polymers to allow for cross-linking (Park et al. 2014). Incorporation of gelatin into hyaluronan increased seeded cell proliferation in a dose-dependent fashion (Park et al. 2014).

Silk

Unlike hyaluronan, silk is a protein-based polymer, produced by insects and spiders. In recent years, silks and silk-derivatives have been studied in tissue engineering as lightweight yet tough biomaterials (Zhang et al. 2015). For this reason, silk has been used to reinforce gelatin scaffolds, resulting in greater tensile and bending strength (Zhang et al. 2015). Gelatin-silk hydrogel composites were also synthesised into hydrogels which gelate upon contact with aqueous methanol (Silva et al. 2014). This was due to the solution inducing transformation of the silk from random-coil to β-sheet conformation, causing physical cross-linking of the hydrogel. These hydrogels were thermally-responsive—when the temperature raised from 20 to 37 °C, their swelling greatly increased and the hydrogels experienced greater mass loss due to gelatin release (Silva et al. 2014).

Other natural polymers

Wang's group invented a phase transfer cell culture (PTCC) system, adopting gelatin as delayed dissoluble porogen in either agarose or alginate hydrogels (Gong et al. 2010; Su et al. 2012). In terms of gelatin as a capsule containing chondrocytes followed by

dissolution leaving cavity filled by cells and their ECM, a phenomenon of directed cell proliferation and sprout out as isogenic groups was observed (Ng et al. 2012). An engineered 3D scaffold-free living cartilage graft was developed for transplantation of therapeutic cells or further study (Lau et al. 2012; Su et al. 2012).

Gelatin blended with synthetic polymers

One of the key strengths of synthetic polymers in tissue engineering is the ability to tune and modify material chemistry (Toh and Loh 2015). However, most synthetic polymers lack inherent cell-recognition sequences and binding sites (Toh and Loh 2015). Therefore, the synergy between gelatin, a protein-based material with many cell-recognition sites, and synthetic polymers can be harnessed for modulating cell adhesion to the scaffolds. For instance, polyamide (PA) coated with gelatin significantly promoted cell adhesion and did not lose biomechanical properties, as well as facilitated wound healing while being transplanted to nude rats (Ulrich et al. 2014).

Poly(lactic-co-glycolic acid) (PLGA)

Gelatin-PLGA composite microparticles were synthesised by emulsifying gelatin in a PLGA/dichloromethane solution and following standard water/oil/water emulsion microparticle synthesis techniques (Ozkizilcik and Tuzlakoglu 2014). The RGDS-containing peptide was attached to the gelatin portion of the microparticles with carbodiimide cross-linking. Chondrocytes cultured on gelatin-PLGA microparticles had higher viability, greater proliferation, and larger deposition of glycosaminoglycans compared to PLGA microparticles alone (Ozkizilcik and Tuzlakoglu 2014). Incorporation of RGDS motifs further increased cell proliferation and viability. This system is also injectable and could potentially be utilized for the delivery of chondrocytes to articular defects.

Another approach to combine PLGA and gelatin is by electrospinning techniques. The unloaded fibres have also been characterized for mechanical properties and their ability to support pre-osteoblasts growth in vitro, where composite fibres featured greater cell adhesion and proliferation than PLGA alone (Meng et al. 2010). In general, incorporation of gelatin into PLGA constructs allows for further adjustment of release kinetics as well as increases cell biocompatibility.

Oligo(poly(ethylene glycol) fumarate) (OPF)

Cross-linked OPF is a polyethylene glycol (PEG)-based hydrogel with cleavable fumarate groups, allowing for biodegradation. The properties of OPF can be controlled by varying molecular weight, cross-linking density, and types of crosslinker molecule. OPF has been demonstrated to support cell encapsulation and tissue regeneration.

To this effect, gelatin microparticles, loaded with either insulin-like growth factor 1 (IGF-1), TGF-β3 or both, were encapsulated in the cartilage layer of a bilayered OPF construct for the treatment of a rabbit osteochondral defect. In other bilayered constructs, the growth factors were loaded directly into the OPF gel phase (Kim et al. 2013). Constructs were able to release growth factors in vitro for at least 25 days. In the animal model, greater frequency of hyaline-like cartilage was observed when TGF-β3 was delivered in the presence of IGF-1 (Kim et al. 2013). Ongoing research on gelatin-OPF composite constructs holds promise for the generation of complex tissues, such as the osteochondral junction.

Gelatin used alone

Apart from coordinating with other polymers, gelatin per se can be directly adopted in tissue engineering. A microcarrier is a typical model. Genipin-cross-linked gelatin microspheres were shown to support the growth of highly viable HepG2 cells and further mediate the spontaneous formation of functional hepatocellular aggregates on differently sized microcarriers (Lau et al. 2011). Another more elegant design is a gelatin-based cell microcarrier with open, hollow and shell-like structure, fabricated by a special surface cross-linking technique and subsequent dissolution of uncross-linked material (Su et al. 2011). The microcarrier showed high cell loading efficiency and good biocompatibility, which can support favourable cell adhesion, rapid proliferation and controllable differentiation.

Gelatin as drug delivery vehicles

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 (Kumari et al. 2010). Thus, another important research direction involves the study of gelatin within composite material systems for the controlled delivery of therapeutics. Composite systems synergistically combine two or more materials in order to produce a new system with novel properties unique to either material alone, such as extended release kinetics or increased mechanical properties.

Anti-cancer drug delivery

Gelatin nanoparticles (GNPs) have been extensively used for the delivery of both hydrophilic and hydrophobic anti-cancer drugs, aimed at sustaining the release, increasing targeting efficiency and minimising toxicity. The benefits of GNPs to deliver anti-cancer drugs include (i) their very low cytotoxicity; (ii) reproducible production that may lead to future upscaling and (iii) the low cost of gelatin. Another important feature is the passive targeting ability of GNPs, through which the nanoparticles remain at the tumour region for sufficient time until complete release of the loaded drugs. These findings were confirmed by the rapid uptake and long-term retention demonstrated by GNPs in the tumour after administration. Paclitaxel-loaded GNPs showed 2.6 times higher bladder tumour tissue concentrations compared with the commercial Cremophor/EtOH formulation after intravesical administration into dogs (Zhang et al. 2014).

The superior efficacy of anti-cancer drug-loaded GNPs compared to free drugs was manifested both in vitro (in cancer cell lines) and in vivo (in tumour-bearing animal models). The tumour volume in mice treated with free 17-AAG was increased 20 times relative to the initial volume, whereas that of 17-AAG/rHG-TOS nanoparticles was elevated 15 times (Won et al. 2011). After intraperitoneal injection of DOX-loaded GA-cross-linked GNPs into rats, the efficiency of DOX was enhanced compared to free drugs; however, high cardiotoxicity was also observed upon repeated administration.

Protein and vaccine delivery

GNPs have also been used to deliver protein and peptide drugs. BSA as a model protein has been investigated for decades. 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 (Tang et al. 2012a). In another study, rHG nanoparticles showed great potential for delivery of FITC-BSA in terms of sustained release, less initial burst, and safety (Won et al. 2012). Other protein drugs including insulin, tissue-type plasminogen activator (t-PA), BMP-2, alkaline phosphatase (ALP) and angiogenic bFGF were successfully encapsulated into GNPs with the biological activity of those protein drugs were retained in vivo.

Miscellaneous drug deliveries

GNPs have also been used for the delivery of a wide variety of drug types including anti-HIV (didanosine), anti-malarial (chloroquine phosphate and cryptolepine hydrochloride), anti-fungal (fluconazole and amphotericin B), anti-tubercular (rifampicin and isoniazid), anti-inflammatory (ibuprofen and indomethacin), analgesic (paracetamol), skeletal muscle relaxant (tizanidine hydrochloride) and oral hypoglycemic (rosiglitazone) agents. In these applications, GNPs exhibited the desired functions such as: (i) *extending drug release;* GNPs released cryptolepine for up to 192 h (Kuntworbe and Al-Kassas 2012); (ii) *reducing side effects;* encapsulation of amphotericin B and cryptolepine into GNPs reduced their nephrotoxic and hemolytic side effects, respectively, compared to the free compounds (Kuntworbe et al. 2013); 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), and improved schizonticidal activity in vivo (Kuntworbe et al. 2013). Also, rifampicin-loaded GNPs resulted in enhanced uptake of drug by the lung tissue, improving its bioavailability and significantly reducing bacterial counts in the lungs and spleen of TB-infected mice (Saraogi et al. 2010).

Drawbacks and challenges

A common inquiry over the use of gelatin as biomaterials is whether this polymer is prone to bacterial infection (Sasaki 2014), given its use as a medium component for bacterial culture (Bezrukikh et al.

2014). To date, there is no solid evidence to support this argument and sterile gelatin is generally considered safe. In addition, several anti-bacterial agents can be employed to further process gelatin (Li et al. 2013). Meanwhile, like other animal-origin proteins, gelatin is also questioned for its risk of carrying harmful agents such as transmissible spongiform encephalopathies (TSEs). However, in the case of gelatin, the rigorous manufacturing processes such as acid, alkaline and heat treatments should be sufficient effective to inactivate TSE (Won et al. 2011). Additionally, recombinant human gelatin (rHG), which is commercially available, non-toxic and low immunogenic, can be an ideal substitute for the natural one (Won et al. 2011). Several companies (e.g. FibroGen South San Francisco, CA, USA) are already producing gelatin by the recombinant DNA technology.

Commercial gelatins used in the pharmaceutical industry are heterogeneous mixtures of differently sized proteins derived from bovine or porcine bones or skins with a wide range of molecular weights producing heterogeneous size distribution. Strategies to overcome this drawback is to apply the two-step desolvation technique, as previously discussed (Ayre et al. 2014), or to use rHG due to its homogeneity in molecular weight and ability to form micro/nanoparticles with narrow size distribution.

Conclusion

Gelatin is rated among top macromolecule candidates for the fabrication of various biomaterials. Its biological activities to support cell adhesion and interact with signalling molecules are desirable for cell delivery and tissue regeneration. Its physicochemical features, in particular the tuneable cross-linking density, degradation kinetics and gelling properties, offers high versatility for the design of drug delivery vehicles. Chemical modification of gelatin further allows for enhanced drug stabilisation and higher drug entrapment efficiency. In addition to its use alone, gelatin has also been successfully incorporated in numerous composite materials for regenerative applications such as musculoskeletal tissue engineering. The synergistic use of gelatin and other biomaterials enables higher flexibility of material degradation and controlled drug release, while maintaining and enhancing the properties of the bulk material (typically a ceramic or other polymer). Overall, the modification of gelatin and its combination with other biomaterials demonstrate its versatile potentials in the fields of drug delivery and tissue engineering.

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