Research & Reviews: Journal of Material Sciences
DOI: 10.4172/2321-6212.1000153

e-ISSN: 2321-6212
p-ISSN: 2347-2278

Chitosan/Poly(γ-glutamic acid) Polyelectrolyte Complexes: From Self-Assembly to Application in Biomolecules Delivery and Regenerative Medicine

Antunes JC[1,2,3], Gonçalves RM[1,2,4] and Barbosa MA[1,2,4]*

[1]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

[2]INEB-Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

[3]Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal

[4]ICBAS-Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

Research Article

Received: 29/08/2016
Accepted: 15/09/2016
Published: 25/09/2016

***For Correspondence**

Barbosa MA, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal, Tel: +351 220 408 803;

Email: mbarbosa@ineb.up.pt

Keywords: Polyelectrolyte complexes, Poly(γ-glutamic acid) , Chitosan, Regenerative medicine, Delivery system

ABSTRACT

Physically complexed Polyelectrolyte (PE) Complexes (PECs) have been widely used for different purposes. Mixing solutions of oppositely charged PEs leads to spontaneous formation of PEC dispersions (complex coacervates) of interpolymer complexes driven by electrostatic interactions. Assembling of poly(γ-glutamic acid) (γ-PGA) with chitosan (Ch) in PECs provides an easy and flexible technology for the delivery of biomolecules in tissue engineering. γ-PGA is an anionic polymer, recently explored but with interesting biological properties, namely non-immunogenicity and in vivo biodegradability into glutamic acid units. Its combination with cationic polymers, namely Ch, is being increasingly investigated. Here the formation of PECs, namely different architectures, as well as detailed features of biomedical applications of Ch/γ-PGA PECs as delivery systems and mediators of tissue regeneration is reviewed. Besides overviewing the literature on Ch/γ-PGA PECs applications, the main challenges to the use of Ch/γ-PGA PECs and the most promising applications of these structures in regenerative medicine are covered.

INTRODUCTION

Development of more advanced and functional biomaterials has been an ongoing challenge in the last decades towards the benefit of millions of patients worldwide that need to be treated with biomaterial-based therapies. The ability of individual polymers to respond to changes in pH, temperature, electric or magnetic fields, together with the possibility of combining different polymers, is a strategy largely pursued in the field [1-4].

Polyelectrolyte (PE) Complexes (PECs) have been particularly under focus, especially as delivery systems of biomolecules [2-5]. Systems that can respond to a dynamic pH environment are of particular interest for applications within different locations in the body that exhibits substantial pH changes during either healthy or diseased state [2]. PECs have the ability to improve intrinsic properties shown by each PE (e.g. cytocompatibility and hydrophilicity [6-10]), enhance bulk properties (e.g. mechanical properties [11,12]) and act as delivery systems with increased capability (e.g. pH-responsiveness [13-17], higher loading efficiency [13,18] and effectiveness [9,18]).

Chitosan (Ch) has been one of the most studied PEs. Ch's chemical structure resembles human polysaccharides that are present in the extracellular matrix of numerous tissues as proteoglycans. Ch is the only naturally occurring cationic glycosaminoglycan (GAG) analogue [1,19-21], prone to molecular recognition by living cells or tissues. It is an efficient delivery vehicle for nucleic

e-ISSN: 2321-6212
p-ISSN: 2347-2278

acids, binding strongly with negatively charged biomolecules, and useful for gene therapy [22-25]. Plus, as a cationic polymer, Ch can be combined with anionic polymers.

Poly(γ-glutamic acid (γ-PGA) is one of the most promisor anionic PEs, highly due to its non-immunogenic behavior [26-28] and biodegradability into glutamic acid residues [29,30]. Glutamate is mostly known as major excitatory neurotransmitter in the central nervous system, acting as a synaptic transmitter, and influencing neuronal excitability, synaptic structure and function, neuronal migration during development, and neuronal viability [31-35]. It is also present in other tissues and organs, specifically in non-neuronal tissues like bone, pancreas, skin and cartilage [31,33-36], and having significant roles in their normal function, although yet not fully understood. γ-PGA, being a poly(amino acid) built by glutamic acid monomers [29,30,37,38], may participate in similar processes.

Ch/γ-PGA PECs appear to be promisor with an increasing number of references in the literature, but there is a lack of systematized information. Therefore, this review aims to overview the process of PEC formation within different architectures, as well as the current status of the Ch/γ-PGA PECs and their applications in the fields of drug delivery and regenerative medicine. Within this scope, we expect to identify the main challenges of Ch/γ-PGA PECs application for tissue regeneration.

CHITOSAN (Ch) AND POLY(γ-GLUTAMIC ACID (γ-PGA) AS POLYELECTROLYTES (PEs)

Polyelectrolyte (PE) is the term used to classify polymeric macromolecules with charged or chargeable groups when dissolved in polar solvents (predominantly water) [39,40]. When a PE dissociates, it gives rise to a macroion and counterions in aqueous solution [39]. The macroion can be named polycation (positively charged PE) or polyanion (negatively charged PE) [40]. **Table 1** highlights some of the most studied PEs, either from natural or synthetic origin.

Table 1. Natural and synthetic polycations, and polyanions, used for polyelectrolyte complex (PEC) assembly [41-48].

	Natural	Synthetic
Polycation	Ch, Col, Gelatin, PLL, Arginine, Starch.	Poly(ethylene imine), Poly(allylamine hydrochloride), PDADMAC, Poly(vinyl pyrrolidone), Poly(amidoamine), Poly(N-isopropyl acrylamide), Poly(2-(dimethylamino)ethyl methacrylate), Poly(2-(diisopropylamino)ethyl methacrylate).
Polyanion	HA, chondroitin sulfate, PGA, Cellulose sulfate, Alginate, Pectin, Heparin, Mucin, DNA, Poly(aspartic acid), Dextran sulfate, CMC, GG.	Poly(acrylic acid), Poly(styrene sulfonate), Poly(vinyl sulfate), Poly(methacrylic acid).

*PEC: Polyelectrolyte Complex; Ch: Chitosan; Col: Collagen; PLL: Poly(L-lysine); PDADMAC: Poly(diallyldimethylammonium chloride); HA: Hyaluronic Acid; PGA: Poly(glutamic acid); DNA: Deoxyribonucleic Acid; CMC: Carboxymethylcellulose; GG: Gellan Gum.

In particular, Ch is a polysaccharide and a linear copolymer of glucosamine and N-acetylglucosamine units connected through a β-1,4-linkage [41-51]. On the other hand, γ-PGA is a poly(amino acid) built by D- and L-glutamic acid residues linked by peptide bonds [29,37]. Both PEs are represented in **Figure 1**.

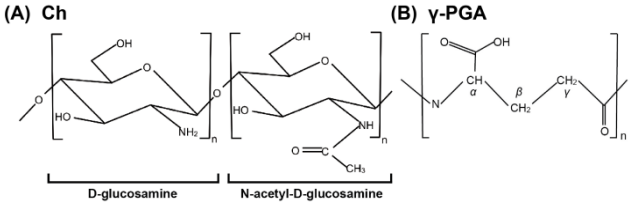

Figure 1. Chemical structure of the molecular units of Ch (A) and γ-PGA (B).

C. Rouget was the first to identify Ch in 1859 [52,53]. Ch is mainly obtained by N-deacetylation of chitin under alkaline conditions [20,54-56]. Chitin is abundant in Nature (primary structural component of the exoskeleton of shrimps, crabs and lobster and squid pens as an equivalent to collagen, and present in lesser amounts in cell walls of some fungi and yeast and in plants as the equivalent of cellulose) [20,54,56-58]. When the degree of acetylation (DA, molar fraction of N-acetylated units) is lower than about 50%, the polymer is termed Ch [58]. Ch's molecular weight (M_w) and DA are its main structural parameters influencing the overall behavior of the polymer as a biomaterial [1,59]. Ch is soluble in nearly all diluted aqueous acidic solutions and insoluble in water, concentrated acid, alkali, alcohol, and acetone and in common organic solvents. The dissolution occurs by protonation of the primary amine ($-NH_2$) groups of the D-glucosamine molecular unit [20,54,56,59,60]. These amine groups become protonated, and the polymer behaves as a PE [61,62]. As a PE, Ch has been extensively screened for its ability to form complexes with other polymers, particularly through electrostatic interactions [1,20,50,57,63].

Ch has been extensively described for a large range of biomedical applications [1,20,53,55,64-66], in numerous structures and forms [51]: 2D films [67-70], PEM films [71-74] or nanostructured surfaces [75]; small-scaled particles like nanoparticles [76-80], microparticles [81-83] or PEM micro- [84-86] or nanocapsules [87-89]; and 3D porous structures such as sponges [90-95], fiber meshes [96-101], or gels [102-106], either alone or in combination with other molecules. Parameters such as biodegradability and biocompatibility are

e-ISSN: 2321-6212
p-ISSN: 2347-2278

particularly important. Ch can be degraded enzymatically, through chemoenzymatic means, recombinant approaches and physical means like electromagnetic radiation and sonication [57,60,107]. In humans, *in vivo* degradation of Ch is thought to be primarily due to the activity of lysozymes (present in articular cartilage, liver, plasma, saliva, tears and milk [108]) and bacterial chitinolytic enzymes (e.g. chitosanase) that have been identified in human tissues of the gastrointestinal tract and lung [54,107,109,110]. These enzymes hydrolyze both glucosamine and acetylated residues, leading to polymer erosion into a suitable size for renal clearance [19,50,58,107]. Ch has additionally been regarded as a non-toxic and a biologically compatible polymer [1,55,56,111]. Ch and its derivatives have also been tested successfully against numerous cell types [54]. Moreover, it is approved by the Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) as a wound dressing material (topical intended use) [61]. Ch has also been shown to possess mucoadhesive [112,113], chemoattractive [114-117], analgesic [118], antibacterial and antifungal [119-121], and hemostatic properties [122,123] (among others).

On the other hand, γ-PGA was firstly detected by Ivanovics and Bruckner [124] in 1937, as the sole constituent of the *Bacillus anthracis* surface capsule, and released into the medium upon bacterial cell lysis. After its discovery, γ-PGA was identified in the growth medium of several *Bacillus* strains as an extracellular product of their fermentation [29,125]. γ-PGA can be characterized by its M_w distribution, the ratio of D- to L-glutamic acid monomers and the carboxyl-group (α or γ) engaged in the peptide bond [29]. γ-PGA is soluble in water [30]; in acidic solutions, the carboxyl groups of γ-PGA become deprotonated [126,127] and the polymer can also be explored as a PE. As a PE, γ-PGA has been object of some studies regarding its ability to form complexes with other polymers, namely through electrostatic interactions [8,128-132].

In the case of γ-PGA, its potential for biomedical applications has been less explored than for Ch [29,30,133,134]. Still, γ-PGA has been processed in the form of 2D films [135,136], or PEM films [71,131,137]; small-scaled particles like nanoparticles [128,138-142], microparticles [143,144] or PEM nanocapsules [129]; and 3D porous structures such as sponges [7,8,11,12,145,146], fiber meshes [147-151], or gels [152-158], particularly in combination with other molecules. γ-PGA can be chemically degraded by prolonged exposure to an extreme pH value at high temperatures, physically by ultrasonic irradiation, or enzymatically [29]. In γ-PGA producing *Bacilli*, endo-γ-glutamyl-peptidase and exo-γ-glutamyl-peptidase are the two types of enzymes that can degrade γ-PGA. In mammals, the existence of γ-PGA has also been reported [159]; being assumed that this polymer can be biochemically degraded into glutamic acid residues *in vivo* [29]. γ-PGA non-immunogenicity feature has been demonstrated by a lack of immune response after repeated polymer injections [26-28], reinforcing a fact already discovered when γ-PGA was shown to mask the dangerous pathogen *Bacillus anthracis* from immune surveillance of the host [26,160-162]. Afterwards, γ-PGA and its derivatives have been tested successfully under different shapes and against some cell lines [6-8,139,145,148,155,163], and primary cells [164,165]. Moreover, since γ-PGA is edible, it is generally regarded as safe [26,30,160,166].

SELF-ASSEMBLY AND ELECTROSTATIC COMPLEXATION OF PEs

Self-assembly is a spontaneous association of multiple components into larger entities [167-171]. Its concept can be enlightened as follows: the 'assembly' connotes 'to put together or build' and the 'self' implies 'without outside help or on its own'. The 'self' that drives the assembly is(are) the interaction(s) between the assembling objects [172]. This process can occur at any scale, ranging from nano- and micrometer scale to macroscopic dimensions [173,174]. Self-assembly is the most ubiquitous process in Nature [171,175-178], being present in many biological systems. Classical examples include the hierarchical self-assembled rod-like structure of tobacco mosaic virus [179-181], phospholipids forming the membranes of cells and organelles [127,182-184], deoxyribonucleic acid (DNA) self-complementary double-helix annealing [127,185], and protein aggregation forming the Extracellular Matrix (ECM) of connective tissues, namely fibronectin and collagen [186-189]. The assembling objects behind self-assembly are active building units bearing recognition information, and thus capable of recognizing each other. If the assembly happens in multiple stages, the building block can be different in each stage [168,190].

The self-assembly process relies on the action of non-covalent interactions (or weak, and also reversible, covalent interactions) between the building blocks, underlying their assembly into larger and thermodynamically stable structures. A balance between attractive and repulsive forces should take place in order to obtain stable self-assembled structures [168,191,192]. The most described forces behind self-assembly comprehend van der Waals forces (including hydrogen bonding), hydrophobic interactions, electrostatic forces, magnetic interactions, aromatic stacking, metal-ligand bonds and/or entropic effects (**Figure 2**) [169,171,172,191,193,194].

Electrostatic forces offer a type of bond that is low demanding in terms of its directionality and the distance between oppositely charged functional groups (it has the least steric demand of all chemical bonds [195-203]), in addition to the possibility of forming multi-centre bonds [204]. Furthermore, the magnitude and length scale of these interactions can be regulated, namely by choosing the solvent (e.g., dielectric constant) and/or the concentration and chemical nature (e.g., size and valence) of the surrounding charged counterparts [191]. Electrostatic self-assembly is an easy, reliable, cheap and versatile example of the known self-assembly processes; ergo, subject of numerous studies up until now [41,173,191,203,205-209].

Numerous building blocks – amphiphiles (lipids and surfactants) [210,211], polymers [212-214], copolymers [215-217], functional molecules/macromolecules (poly(amino acids) [218], peptides [219], proteins [220,221] and DNA [222,223]), nanoparticles (from biological origin – charged viruses [224] – to inorganic – magnetic, gold or silver [225-228]) – have been assembled by electrostatic interactions

e-ISSN: 2321-6212
p-ISSN: 2347-2278

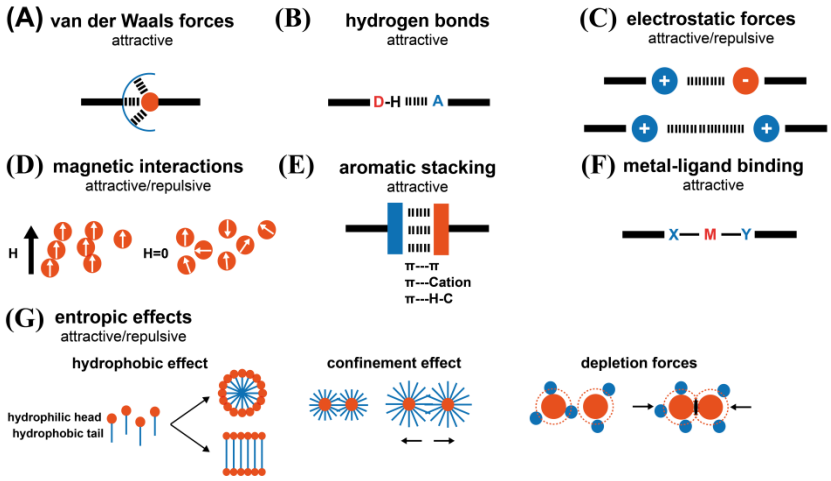

Figure 2. Forces involved in self-assembly. (a) Van der Waals forces; (b) hydrogen bonds between a H-bond donor and a H-bond acceptor; (c) electrostatic interactions between oppositely, or likely, charged species; (d) magnetic interactions in response to a magnetic field (H); (e) aromatic stacking with face-to-face or edge-to-face interactions; (f) metal-ligand binding between a soluble metal acceptor centre (M) and organic ligand donors (X and Y); and (g) entropic effects, namely hydrophobic effects (separation of hydrophobic parts of amphiphilic objects from water molecules), confinement effects (increase of steric repulsion) or depletion forces (mediated by smaller particles or solvent molecules). Figure adapted from [171,190,191,195-202].

[199,207,209]. Although not complete, this list demonstrates the diversity of building blocks that can be used. Different self-assembled shapes have been obtained, as a result of the geometries of all interactions elicited by that object [168]: two-dimensional (2D) films, small-scaled particles or three-dimensional (3D) structures [168,199,207,209,229]. Examples of such outlines are represented in **Figure 3**.

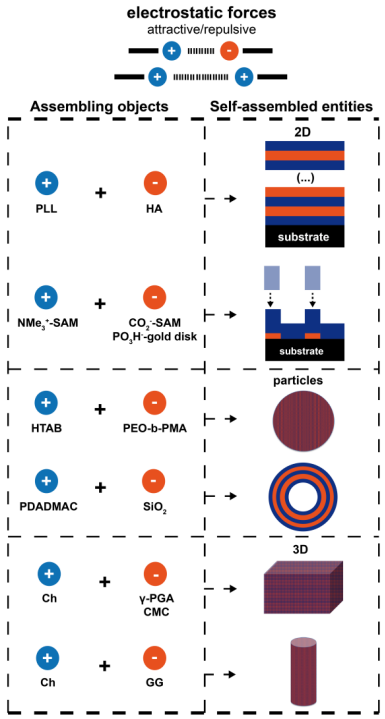

Figure 3. Self-assembled entities obtained from oppositely charged self-assembling objects. 2D films: cationic poly(L-lysine) (PLL) and anionic hyaluronic acid (HA) alternate deposition onto a planar substrate; and planar and curved patterned gold substrates (NMe_3^+-terminated- or, CO_2^--terminated self-assembled monolayers (SAMs)), coated with PO_3H^--terminated gold disks. Small-scaled particles: nanoparticles composed by the block copolymer poly(ethylene oxide)$_{210}$-b-poly(tert-butyl methacrylate)$_{97}$ (PEO-b-PMA) and oppositely charged surfactant hexadecyltrimethylammonium bromide (HTAB); and hollow layer-by-layer (LbL) capsules containing cationic polymer poly(diallyldimethylammonium chloride) (PDADMAC) and anionic silicon dioxide nanoparticles (SiO_2). 3D structures: freeze-dried scaffolds composed of cationic chitosan (Ch), and anionic poly(γ-glutamic acid) (γ-PGA) and carboxymethylcellulose (CMC); and microfibers containing cationic Ch and anionic gellan gum (GG). Figure adapted from [212,230-235].

PECs in Solution

Molecules aggregate with each other in solution [236]. In general, mixtures of two neutral polymers (or PEs with same/similar charge density) in solution present thermodynamic incompatibility, with phase separation occurring when the polymer concentra-

e-ISSN: 2321-6212
p-ISSN: 2347-2278

tion increases and the different phases formed become enriched in one of the components. However, when PEs present opposite charges, electrostatic interactions are established between them. When phase separation occurs, a phase becomes enriched in the two polymers while the other is composed by solvent only [237]. Hence, phase separation results in macroscopic phase separation, or in polymeric complexes [238].

Bungenberg de Jong [236,238] was the first to suggest PEC formation in solution, between gelatin and gum arabic, by observing the latest aforementioned phenomenon. Such phase separation occurrence was named "complex coacervation", and the polymer condensed liquid phase "complex coacervate" [236]. Therefore, complex coacervation is the spontaneous process that results in the aggregation of macromolecules or formation of colloidal particles under the action of electrostatic attractive forces [239,240]. Fuoss [241-243], Michaels [40,208,244,245], Kabanov [246-248], Tsuchida [236,249,250] and Dautzenberg [215,251-253], among others [236], were pioneers in the study of this method with PEs in solution, and at the colloidal level. Mixing solutions of polycations and polyanions lead to spontaneous formation of PEC dispersions (complex coacervates) of interpolymer complexes [236,254,255].

PECs in 2D: PE Self-Assembly into Multilayers (PEMs)

The technique known by layer-by-layer (LbL) self-assembly is considered the most well established bottom-up assembly methods of thin film deposition [41], and widely used for 2D PEC formation. With this method, PEs can be self-assembled by sequential adsorption of multiple thin polyion films from aqueous solutions onto charged solid substrates, forming PE Multilayers (PEMs) (**Figure 3**) [170,203,213,256].

Multilayer formation was first suggested by Kirkland [257] and Iler [258] in 1965-1966 [41,209,259]. They described for the first time the formation of layers of charged particles by adsorption from solution, by alternating deposition of positively and negatively charged colloid particles, such as silica and alumina. However, it was Iler [258] that suggested a way to prove the existence of a multilayered structure by use of reflected light after each layer addition. Still, Kirkland patented the system in 1970-1984 [260,261]. In 1980 [262], 1982 [263], 1984 [264] and 1990 [265] other researchers started suggesting multilayered film assembly based on electrical charge reversal after each deposition step. In 1991, Decher and Hong [210,266] analyzed the thickness of multilayered films built by anionic and cationic bipolar amphiphiles and suggested the use of PEs for PEM formation from sufficiently concentrated PE aqueous solutions. Layer numbers of 35 and 39 were achieved, with corresponding 170 and 151 nm of thickness, calculated through small angle X-ray scattering.

Regardless, only in 1992, PEM formation through electrostatic interaction exclusively between PEs was reported [213]. PEM films of 100 consecutively alternating layers were assembled [203,213]. Through this new technique, a solid substrate with a positively charged planar surface (silanized aminopropylsilanized fused quartz or silicon single crystal substrates) was immersed in a solution containing an anionic PE (poly(styrene sulfonate)) and a monolayer of the polyanion was adsorbed [213]. Since the adsorption was carried out at relatively high concentrations of PE, a large number of ionic residues remained exposed to the interface with the solution and thus the surface charge was effectively reversed. After rinsing in pure water, the substrate was immersed in the solution containing a cationic PE (poly-4-vinylbenzyl-(N,N-diethyl-N-methyl-)-ammonium iodide). Again, a monolayer was adsorbed and the original surface charge was restored. By repeating both steps in a cyclic fashion, alternating multilayer assemblies of both polymer pairs were obtained. A linear increase of film thickness with layer number was observed. It was technically possible to calculate a PEM thickness of 42.5 nm of 39 deposited layers composed by the PE pair of poly(styrene sulfonate) and poly(allylamine hydrochloride) [213]. This process was designed by (LbL) deposition by Decher and colleagues [203,213]. Since then, surface modification via the LbL deposition technique through electrostatic interactions gained motion. An adaptation of this technique is represented in **Figure 4**.

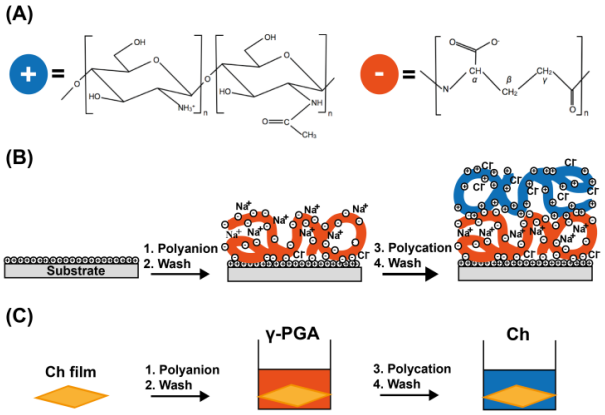

Figure 4. (a) Molecular units of both polymers used in the polyelectrolyte multilayer (PEM) buildup: Ch (left), as a polycation, and γ-PGA (right), as a polyanion. (b) Simplified molecular picture of the first two deposition steps, illustrating film deposition starting with a positively charged surface. Counterions are included. Polyion conformation and layer intermingling are omitted for clarity. (c) Schematics of the layer-by-layer (LbL) deposition process, by horizontal immersion, using gold-coated silicon wafers as substrates, coated with a Ch film, obtained by spin-coating. Steps 1 and 3 represent the adsorption of the γ-PGA and Ch, respectively. Steps 2 and 4 are the intermediate rinsing with buffer solution. With such cyclic depositions, the simplest multilayer film architecture can be obtained. Figure reprinted with permission from Antunes JC, *et al*. [71]

e-ISSN: 2321-6212
p-ISSN: 2347-2278

A solid substrate with a positively charged planar surface is immersed in the solution containing the anionic PE and a monolayer of the polyanion is adsorbed (step 1 or step A). Since the adsorption is carried out at relatively high concentrations of PE, a number of ionic groups remain exposed to the interface with the solution and thus the surface charge is reversed. After rinsing in a buffered solution (or in pure water) the substrate is immersed in the solution containing the cationic PE. Again a monolayer is adsorbed but now the original surface charge is restored (step 3 or step B). These four steps are the basic buildup sequence for the simplest film architecture, $(A/B)_n$, since by repeating the steps in a cyclic fashion (A, B, A, B, ...), alternating multilayer assemblies of both polymers are obtained [213]. In more detail, LbL film-deposition requires a solid support bearing a minimal surface charge to initiate the assembly process. If not inherent, the substrate surface charge can be created by chemical or physical modifications. Cleaned hydrophilic glass, quartz, silicon wafers, mica, and gold-coated supports are the most frequently used materials. They are typically chosen according to their convenience for particular analytical methods [209,256,267]. The structure and properties of each layer of PE are typically independent on the substrate used after a few deposition cycles, being then governed by the choice of the respective polyanion/polycation pair and by the deposition conditions [259]. Self-repulsion of the already deposited layers should also take place, to adjust molecules conformation and consequent flexibility of the underlying layers, and promote further PE deposition [170,267]. Layer intermingling, imposed by intrinsic charge compensation [268], is also frequent [170,267].

Several important parameters influence PEMs buildup and need optimization for each PEM. They include: pre-treatment of the substrate, nature of the PEs, concentration and temperature of the solutions, solvent, salt type and ionic strength, pH, dipping time, rinsing time, agitation during adsorption or rinsing, drying step, and environmental conditions such as temperature and humidity of the surrounding air [209,256,267,269,270].

The main advantages of PEM formation through LbL self-assembly technique derives from: i) its simplicity, since no complex equipment is required for the buildup (a suitable substrate, container(s) compatible with the materials used and a substrate handling tool [209]); ii) each adsorption step is relatively quick (typically 10-20 min/adsorption step, but can be between 1 min and 1 h due to PE adsorption kinetics [221,231,256,269,271,272]); iii) low costs [170,273,274]; iv) compatible with industrial production methods [231,275]; v) easy build-up [231,256,267]; vi) the possibility of introducing different molecules [203,209,256,259,276], into the multilayer in a programmed sequence, adding new functionalities [170,231,275,276]; vii) the possibility of controlling PEC architecture at the nanometer-level, by selecting the substrate and building blocks, layering sequence, experimental conditions and influencing parameters [203,231,256]; viii) the lack of need for auxiliary molecules, such as catalysts or initiators, to initiate the reaction (generally performed in aqueous solution), which represents the main advantage over covalently crosslinked networks, favoring biocompatibility thus avoiding purification before administration [1].

The main limitations of PEMs include: i) fuzzy nano-assemblies, derived from the intermingled structures fabricated from LbL technique [170,203]; ii) interpolymer orientation or alignment of polymer chains in a given layer may be difficult to control [208,269,277-279]; iii) long dipping times in the presence of biological materials can be prone to denaturation or photobleaching; or iv) eventual contamination by previous solutions of the layering scheme [209].

PEMs can be built not only on planar substrates, but also on substrates with different shapes, like protein aggregates, enzyme crystals, colloidal particles, cylindrical systems and even living cells [170,209,270,273,274]. Additionally, when using two building blocks, PEM preparation only requires the two PEs carrying complementary charges [1,203,256]. An exact positional matching of the charged groups is also not required [274]. Regardless, technical advances of this technique, which include the use of automated dipping robots [269,280,281], hydrodynamic LbL (with moving substrates [269,282], or solutions [283-285]), spin-assisted LbL [286-288], spray-assisted LbL assembly [289-292], ink-jet assisted LbL [293,294], among others, are assisting in overcoming PEMs limitations, while increasing its use [41,209]. Combination of the bottom-up LbL technique with top-down lithographic techniques [295-298] are another valuable possibility [41].

PEM films have been mainly studied as potential coatings for implantable biomaterials, namely to inhibit coagulation, inflammation or infections (choosing anti-thrombogenic, anti-inflammatory or anti-bacterial PEs, for instance), non-fouling properties (to prevent immune rejection or to enhance the efficiency of injected delivery vehicles), increase protein adsorption and/or promote integration with native tissue (having adhesive proteins or ECM components as building blocks, among other possibilities), cell/biomolecules encapsulation and preservation of their functionality (parameters such as wall thickness and permeation can be modified according to the needs of the system), localized delivery of different molecules in response to different stimuli (namely pH-responsiveness), and modification of substrates to influence cellular processes as adhesion, proliferation, differentiation and ECM synthesis/degradation (tuning PEMs biodegradability, mechanical properties and bioactivity, either inherent to the PEs or induced by added molecules) [42,45,299-301]. Thin-film biosensors (incorporating able molecules within PEM structure – enzymes, antibodies, proteins, nucleic acids, inorganic colloids or organic molecules) are also important fields of application [301].

Ch/γ-PGA PEMs have been proposed as model surfaces to use as delivery systems (**Table 2**). For example, the chemokine stromal-derived factor-1 (SDF-1) was incorporated into Ch/γ-PGA PEMs using different approaches. Ch/γ-PGA PEMs with SDF-1 were able to increase the recruitment of human bone marrow-derived mesenchymal stem/stromal cell (hMSC) *in vitro* due to a sustained SDF-1 release from the PEMs structure. This highlighted Ch/SDF-1/γ-PGA PEMs as potential hMSC attractants, clinically relevant for different clinical settings, from tissue regeneration to immunomodulation [302]. This strategy inspired the use of

e-ISSN: 2321-6212
p-ISSN: 2347-2278

Ch/γ-PGA PEMs as protein reservoirs to other molecules. For example, the pro-inflammatory cytokine interferon-γ (IFN-γ) was incor

Table 2. Ch/γ-PGA PECs in regenerative medicine: 2D PEMs.

Ch/γ-PGA PEMs							
Ch		γ-PGA	Molecule incorporated	In vitro	In vivo	Main achievements	Ref.
M_w (KDa)	DA (%)	M_w (KDa)					
200	-	1230	-	-	-	First PEM formation with organic solvents	[131]
650, 1200 and 1400	-	600 and 1230	-	-	-	Successful PEM formation with organic solvents, particularly with higher M_w of γ-PGA	[302]
324	10	1050	-	NIH3T3 cell line	-	No cytotoxicity	[71]
324	10	1050	SDF-1	hMSCs	-	Chemoattractor-delivery system for SDF-1	[302]
366	14	1050	IFN-γ	Human monocytes	-	IFN-γ delivery system able to modulate macrophage phenotype (M2 into M1) and counteract cancer cell invasion	[303]

*2D: Two-Dimensional; PEM: Polyelectrolyte Multilayer; M_w: Molecular Weight; DA: Degree of Acetylation; NIH3T3: Mouse Embryonic Fibroblast; SDF-1: Stromal-Derived Factor-1; hMSCs: Human Bone Marrow-Derived Mesenchymal Stem/Stromal Cells; IFN-γ: Interferon-γ.

porated into Ch/γ-PGA PEMs and IFN-γ-PEMs were capable of counteracting human M2 macrophage phenotype while decreasing human cancer cell invasion, pointing the potential of this therapeutic strategy to target macrophages at the tumor site and hamper their cooperation in cancer cell-related activities such as invasion [303].

PEC of different polymers can be proposed, such as poly(Styrenesulfonate) (PSS) and poly(Allylamine Hydrochloride) (PAH), two synthetic polymers initially studied by Decher *et al*. [213] and one of the most widely studied PE pairs, namely to further analyze PEM formation [304-307]. Nevertheless, studies with natural polymers have been preferred for biomolecules delivery and regenerative medicine, since biological recognition allows the PEs to become bioactive and biodegradable [19,58,308,309]. Within natural polymers, focus has been given to Ch, collagen, hyaluronic acid and chondroitin sulfate [221,231,310,311], mostly to act as ECM or potential ECM analogues. For example, the work of Thierry *et al*. [74] with Ch and hyaluronic acid as PE counterparts to release nitric-oxide-donor sodium nitroprusside: loaded PEMs reduced platelet adhesion and enhanced thromboresistance, while promoted the anti-inflammatory and wound healing properties of both PEs. Moreover, with the long-lasting delivery of matrix-bound recombinant human bone morphogenetic protein 2 (rhBMP-2) [310], PEMs could further assist tissue-engineered constructs or metallic biomaterial surfaces, to promote tissue formation or implant integration within the native tissue.

PE Self-Assembly into Patterned Surfaces

As aforementioned, electrostatic interactions can be used to direct the patterning of surfaces through selective electrostatic attachment [233]. This can be achieved by generating charged surface patterns at different substrates based on different lithographic methods, followed by adsorption from solution of molecules carrying the opposite charge [41,233,312-314]. As illustrated in **Figure 3**, charged gold particles can selectively attach to regions presenting the opposite charge, when stirred in suspension with a surface patterned with charged self-assembled monolayers (SAMs). Electroplating gold into cavities of patterned photoresist, releasing the gold into ethanol, and modifying the surfaces with charged alkanethiols, can create charged gold particles with well-defined shapes; various sizes and shapes can be tailored by altering the photoresist pattern [233].

Micropatterning with PEMs can be used to build biosensors, which can be used for immunoassays, drug screening, and tissue engineering (TE) [299]. On the other hand, research on PE micropatterning alone has been directed to produce nanostructured coatings with complex patterns or guide area-selective patterned assembly, with uses mostly found within flexible transistors, sensing elements, integrated circuits, antennas, as well as biomedical implants and other devices [41].

PE Self-Assembly into Small-Scaled Particles

PEs can also spontaneously self-assemble in the form of small-scaled particles, namely at the nanometer scale with a size ranging from 1 to 1000 nm [315-317], by direct mixture of polycation and polyanion aqueous solutions [172,255,318,319].

The process of PEC formation of nanoparticles can also be influenced by several parameters. In what concerns PE counterparts, PE type, M_w and charge density, have to be defined first. Then, media parameters such as concentration of PE solutions, mixing ratio of charged units, salt concentration, pH, temperature and volume of all solutions, must be selected. Finally, while preparing the nanoparticles, the order of PE addition (polyanion to polycation, or vice-versa) as well as questions related to the mixing procedure (mixing type, protocol and device) should be taken into consideration (although typically PE solutions are mixed with a magnetic stirrer at a fixed velocity) [240,318,319]. Environmental conditions, such as temperature and humidity of the surrounding air, are also probable influences in the assembly [256,267,270].

e-ISSN: 2321-6212
p-ISSN: 2347-2278

PEC formation into nanoparticles is usually determined by a fast kinetics (values as low as 5 μs have been described) [255,318], including the initial diffusion stage of mutual entanglement between polymers under release of counterions, formation of primary particles, and the further stage of their aggregation and rearrangement of the already formed aggregates [238,255,318]. This process is illustrated in **Figure 5**.

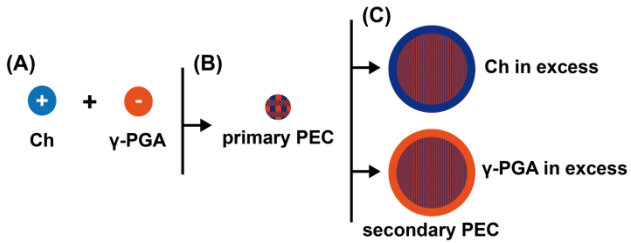

Figure 5. Simplified schematic representation of the process involved in the formation of PEC nanoparticles (a) Representation of the PEs used in the assembly: Ch as a polycation and γ-PGA as a polyanion. (b) Formation of primary complexes comprising a few self-assembled PEs. (c) Formation of secondary complex particles, with a typical core/shell structure, in which the shell contains an excess of one of the PEs. Figure adapted from [320].

After the mixture of the PEs, the dispersion mostly consists of three components: (i) small soluble primary complexes comprising a few polycations and polyanions, (ii) dispersed colloidal particles of aggregated primary complexes (secondary complexes), and (iii) larger insoluble precipitated particles. The former typically exhibit sizes of a few nanometers, the second exhibit sizes of 20-500 nm, and the latter exhibit sizes of 0.1-1 mm. The secondary complex particles (case ii) are the ones with most interest to a clinical setting. They typically form a core/shell structure, according to which a 1:1 charge stoichiometry prevails in the particle core, and in the shell the excess PE component is located, giving the particle the defined charge sign [254,319-321]. Even if the stoichiometry of the charged units is 1:1, the major component may be bound in excess [255]. They can have spherical, rod-like, or toroid shapes, and a loose gel-like up to compact internal structure [319].

The attachment of a PEC colloidal particle is mainly caused by the strong electrostatic interactions between the PE assembling units, whereby the gain in entropy due to the release of low molecular counterions has a major role [255,316,322-324]. Moreover, as for any colloidal particle, a decrease in interfacial energy is critical for its formation [324]. Repulsive forces (**Figure 2**) are also commonly present and may assist in the stabilization of PEC structures. Other attractive forces may additionally take part in the assembly process [255,318].

As for PEMs, PEC formation into nanoparticles is a simple and versatile process, which uses mild conditions, and allows the incorporation of other molecules (drugs, genes, proteins, vaccines, or diagnostic agents) within their structure [47,318,319,325-327]. Control over size, shape and surface chemistry is also a possibility [319]. However, the use of PEC nanoparticles present some advantages over PEM films as: i) absence of substrate restrictions [328]; ii) high surface/volume ratio [321]: small enough to pass through biological barriers [327], internalize into target cells [4] and influence a number of cellular processes [329-331]. Current limitations of PEC nanoparticles include difficulties in the reproducibility of the preparation protocol, size and shape uniformity, conservation of colloidal stability after binding of compounds and interaction with surfaces [240,321].

A higher control over all parameters influencing PEC assembly into nanoparticles is the most described strategy to overcome all limitations [238,240,332-335]. Post-treatment of the dispersions can be applied as well. A known example comprehends consecutive centrifugation, separation and re-dispersion steps of the coacervate phase, which can increase the monomodality of the complexes [214,319,336]: the initial raw dispersion can be centrifuged, the supernatant discarded and the formed coacervate phase of the serum dissolved again to the original volume. The latter is able to increase the amount of secondary complex particles and reduce the polydispersity of the dispersion. Other authors [323,337] filtered the raw dispersions, aiming at the removal of larger aggregate particles and collection of smaller particles towards a lower dispersion of particle sizes [319]. The interaction with surfaces can also be improved by adding molecules specifically to that effect. In the work of Hadju *et al.* [338], folic acid was covalently attached to γ-PGA, Ch/γ-PGA PEC nanoparticles were prepared, and were then able to target folate receptors overexpressed in tumor cells. Loaded with the magnetic resonance imaging (MRI) metal, gadolinium, the nanoparticles showed an increased efficiency as MRI contrast agents. A resume of the main applications of Ch/γ-PGA nanoparticles is described in **Table 3**. As small-scaled particles, Ch/γ-PGA PEC nanoparticles have already been studied as a diclofenac-delivery system and were shown to reduce human macrophage activation *in vitro* [339]. An increased protection from immune surveillance was also shown by Moon and colleagues using Ch/γ-PGA PEC nanoparticles [340]: intranasal administration of recombinant influenza hemagglutinin antigen, or inactivated virus, loaded into Ch/γ-PGA nanoparticles protected mice against the highly pathogenic influenza A H5N1 virus. The safety of γ-PGA was highlighted in addition to its potential to be used as a vaccine adjuvant, not only against influenza, but also against other viruses.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

Table 3. Ch/γ-PGA PECs in regenerative medicine: small-scaled particles.

Ch/γ-PGA PECs in small-scaled particles							
Ch		γ-PGA	Molecule incorporated	In vitro	In vivo	Main achievements	Ref.
M_w (KDa)	DA (%)	M_w (KDa)					
20	15	-	Lansoprazole	-	-	First description of Ch/γ-PGA microparticle preparation	[143]
400	≤10	1230	Antimicrobial peptides LL-37 and NO	-	-	First description of Ch/γ-PGA microparticles as multifunctional carriers for LL-37 and NO	[144]
50	15	160	-	Caco-2 cell line	-	Intestinal delivery systems	[139]
80	15	60	Insulin	Caco-2 cell line	Diabetic male Wistar rats. Oral intake.	Oral insulin delivery	[13]
15	-	>2000	rHA antigen or inactivated virus	-	Female BALB/c mice, seronegative for influenza A virus. Intranasal vaccination	Intranasal mucosal adjuvant for influenza A vaccine	[340]
320	12	1200	-	-	-	First PEC nanoparticle preparation with pH adjustment before self-assembly	[128]
320	12	1200	Gadolinium	HeLa cell line	CD1 female nude mice. MRI imaging in tumor-bearing mice.	Injectable MRI-targeting contrast agents for early cancer detection	[338]
Low	-	-	Doxorubicin	HN-5a cell line	-	No cytotoxicity	[6]
324	11	10-50	-	Primary human macrophages	-	No cytotoxicity	[38]
324	10	10-50	Diclofenac	Primary human macrophages	-	Suppress local inflammation	[339]
60	15	100	DTPA	-	Wistar rats. Study absorption enhancements, after in situ intestinal closed-loop technique	Injectable treatment to improve insulin absorption	[341]
80	15	20	siRNA	HT1080 cell line	-	Cell transfection	[9]
600	20	1000	FITC-labeled dextran	-	-	First PEM nanocapsule preparation. Delivery systems in acidic environments	[129]
10	10	200-500	Insulin	L929 cell line	-	PEM nanocapsules. Cytocompatible. Glucose-triggered insulin delivery	[342]

*NO: Nitric Oxide; Caco-2: Human Epithelial Colorectal Adenocarcinoma; rHA: Recombinant Influenza Hemagglutinin; HeLa: Human Cervical Carcinoma; MRI: Magnetic Resonance Imaging; HN-5a: Human Oral Squamous Cell Carcinoma; DTPA: Diethylene Triamine Pentaacetic Acid; siRNA: Small Interfering Ribonucleic Acid; HT1080: Human Fibrosarcoma; FITC: fluorescein Isothiocyanate; L929: Mouse Fibroblast.

PE Self-Assembly into PEM Nanocapsules

PEM nanocapsules is another spherical architecture at the nanoscale that has been actively studied in the field [3,129,230,341-343]. Colloid-templated electrostatic LbL self-assembly starts with the selection of a colloidal particle template such as silica or polysterene latex particles. Anionic PEs can subsequently adsorb to the charge particles and initiate the LbL self-assembly process, as thoroughly described in section 3.2. Hollow nanocapsules can be obtained after removal of the core particles through their dissolution or decomposition [230].

The process of PEM nanocapsule formation combines the advantages of both PEM films along with the benefits of a spherical shape at the nano-scale, leading to increased control over their size and membrane thickness, in comparison to PEC nanoparticles alone [5,129,344].

The low toxicity, ability to incorporate different molecules, increase load efficiency and stability, control load release and the small size and shape of PEC nanoparticles are giving added value as contrast agents in biomedical imaging, signaling molecules that enhance diagnostic ability, carriers for drug/gene/protein/polynucleotide delivery, and themselves as therapeutic elements [316,319,327,345]. PEM nanocapsules are promising candidates for more complex tasks of storage, encapsulation and release [5]. Main reasons comprise the ability to readily tailor their properties, namely their size, membrane thickness, composition, surface functionality, permeability, and colloidal stability [5,129,344]. Moreover, the stepwise formation of the PEM nanocapsules allows the introduction of multiple functionalities [344], similarly as previously described for the PEM films.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

PECs in 3D Hydrogels

During complexation, PEs can either coacervate, or form a more or less compact hydrogel: macromolecular networks swollen in water or biological fluids [1]. These hydrophilic polymer networks have a high affinity for water, thus high water content [48,346], but are prevented from dissolving due to their physically crosslinked network. As such, water or biological fluids infiltrate the polymer chains of the polymer network, leading to swelling and the formation of a hydrogel [1,346].

After the pioneering work of Wichterle and Lim in 1960 about crosslinked 2-Hydroxyl Methacrylate (HEMA) hydrogels [347], and knowledge of their hydrophilic character and potential to be biocompatible, hydrogels are a thriving research field [2,348,349]. Hydrogels can assume different shapes and reach macroscopic dimensions, depending of the processing methodology employed [1,349,350]. In particular, the formation of 3D PEC hydrogels combines the features of PEs in aqueous solutions with processing methodologies at the macroscale, namely self-assembled gels [351-353], porous scaffolds [7,10,354], microfibers [212,355], or nanofibers [356,357].

The spontaneous gelation into 3D porous PEC scaffolds is one of the most common methodologies actually studied in the field. Main steps of the processing methodology are represented in **Figure 6**.

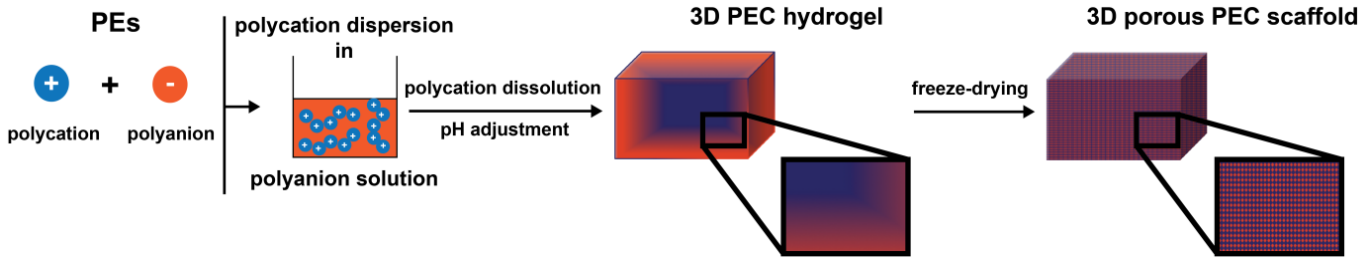

Figure 6. Simplified schematic representation of the process involved in the formation of 3D porous PEC scaffolds. Representation of the PEs used in the assembly: Ch as a polycation and γ-PGA as a polyanion. (b) Figure adapted from [2,71,349].

Formation of 3D porous PEC scaffolds typically starts by blending PE solution with the powder of its PE counterpart to obtain homogeneous dispersion of the latter component. Dissolution and pH adjustment is followed. Spontaneously, charges are mixed at the pH-range of interaction and gel formation occurs [7,10,349,354]. Water-filled 'voids' or 'macropores' can also be formed [349]. Subsequently, the mixture can be cast into shape-forming molds [358], followed by freeze-drying [7,10,354]. pH-responsive 3D porous scaffolds are ergo obtained, structures able to respond to perturbations in the environmental pH through swelling/deswelling, as water is either absorbed or expelled from the hydrogel network [2].

Factors common to all PEC self-assemblies also apply to 3D porous PEC scaffolds, with the main consequence being the degree of PE interaction. Within these structures, global charge densities, PE concentration and relative proportion in the PEC, pH, temperature, ionic strength and order of mixing are the main studied parameters, together with parameters specific of each particular PE [1,349]. Moreover, physical hydrogels such as these are typically reversible and it is possible to dissolve them by changing environmental conditions such as pH and the ionic strength of solution [2,349].

PECs in 3D present some advantages as: i) no need to remove residues of toxic crosslinkers; ii) reversible sol-gel transitions with high sensitivity under mild conditions; iii) convenient incorporation of multiple properties to introduce heterogeneity for hosting hydrophobic drugs [48]. However, we cannot exclude that hydrogels in general tend to possess low mechanical strength, posing significant difficulties in handling, and their sterilization can also be challenging [349].

With 3D porous PEC scaffolds, the control of all parameters influencing their formation, working in consonance to reach the final PEC structure, remains an additional difficult task. However, preparing PECs under reproducible conditions is one strategy that is being attempted. For instance, and as aforementioned in the description of the processing methodology, PE solutions can be mixed at a pH value where complexation does not occur in order to obtain a homogeneous mixture. Only then the pH of the solution can be adjusted to the intended value, thereby promoting the electrostatic interaction [1]. The latter strategy has been found within PEC formation at the macroscale [7,10,354]. Still, physical hydrogels can be heterogeneous, due to clusters of molecular entanglements, or hydrophobically- or ionically-associated domains, which can create inhomogeneities. Free chain ends or chain loops also are also included in the transient network defects of physical gels such as these [349].

pH-sensitive hydrogels based on PEs have additionally been designed to possess precise responsiveness by exploiting the wide pH range of different sites of the human body in order to achieve controlled site-specific biomolecules delivery. PECs pH-responsive hydrogels can be applied in the biomedical field as delivery systems, cell encapsulation, tissue regeneration and wound dressing (within specific applications with low mechanical demands) (**Table 4**) [1,2,48,348,349]. Hydrogel swelling and release profiles can be modulated by tailoring processing parameters [1].

e-ISSN: 2321-6212
p-ISSN: 2347-2278

Table 4. Ch/γ-PGA PECs in regenerative medicine: 3D structures.

Ch/γ-PGA PECs in 3D							
Ch		γ-PGA	Molecule incorporated	In vitro	In vivo	Main achievements	Ref.
M_w (KDa)	DA (%)	M_w (KDa)					
400	10	2000	-	ROS cells	-	First Ch/γ-PGA 3D PECs. Cytocompatible	[7]
400	15	-	-	NHDF cell line	-	No cytotoxicity	[8]
30 and 300	3	1250	-	NIH3T3 cell line	-	No cytotoxicity. Promotion of cell proliferation	[10]
300	3	1250	-	-	ICR male mice. Observation of wound closure.	Wound dressing material, with adequate moisture and increased healing capacity	[12]
300	2-3	1250	-	-	Male Wistar rat. Evaluation of socket healing following tooth extraction	New bone formation in the alveolar socket following tooth extraction	[11]
*3D: Three-Dimensional; ROS: Rat Osteosarcoma; NHDF: Human Normal Human Dermal Fibroblasts.							

As PEC hydrogels, Ch and γ-PGA have been shown to be cytocompatible towards fibroblasts and promote cell proliferation [10], improve wound closure rate and wound healing *in vivo* in mice [12] and promote an earlier and increased bone tissue formation in the alveolar socket following tooth extraction in rats [11]. Interestingly, Chang and colleagues [11] observed a decrease of inflammatory cells presence in the sockets of PEC-treated groups in their histological findings. Moreover, PEC hydrogels led to a larger presence of mineralized tissue, exhibiting significantly earlier as well as greater amounts of new bone formation than treatment with Ch alone. This fact was also attributed to the presence of γ-PGA in the treatment performed. Another possible explanation to this outcome is the possible activation of the glutamatergic signaling, which is functional in bone and results in increased bone formation [35,359]. It is expected that γ-PGA might be consumed/degraded, thus generating a glutamate pool able to activate glutamate signaling pathway [359], as glutamic acid-rich sequences are involved in the nucleation of hydroxiapatite by bone sialoproteins [360].

CONCLUSION

In conclusion, Ch/γ-PGA PECs can be useful for numerous applications. Their cytocompatibility, their immunomodulatory capacity [302,303,339], their stimuli responsiveness [128,129], the ability to control their hydrophilicity and swelling ratio [8,129], together with their versatility to be used as vehicles for drugs, genes, vaccines, enzymes and/or other biomaterials [9,129,144,340] and furthermore promote new tissue formation [11,12] are strong aspects that Ch/γ-PGA PECs have a role in restoring tissue homeostasis.

In truth, PEMs can be successfully produced with many PEs, either biostable or degradable/biodegradable, synthetically produced or biologically derived. But Ch/γ-PGA PECs importance in the regenerative medicine field can have a particular impact. For example, these PECs appear to play a significant role in cartilage tissue, as γ-PGA revealed to promote chondrogenic ECM by hMSCs [164] and enhance type II collagen deposition in an injury model of intervertebral disc [361]. Moreover, γ-PGA-based PECs in proper forms like films, particles, capsules and scaffolds can be used as carriers of different biomolecules to several purposes: SDF-1 [302] to recruit hMSCs, IFN-γ [303] to polarize macrophages, diclofenac [339] to control inflammation, gadolinium [338] to detect carcinogenic features; recombinant antigens or inactivated viruses [340] for vaccines; small interfering ribonucleic acid (siRNA) [9] to increase cell uptake and biological effect; insulin [13] or Diethylene Triamine Pentaacid Acid (DTPA) [341] to increase insulin bodily levels. The non-immunogenic feature of γ-PGA has been opening new perspectives on their use as immunoadjuvant in vaccines and is expected to increase in the field of immunotherapy.

Still, the control of all parameters influencing PEC formation, working in consonance to reach the final PEC structure, together with a high-throughput method of fabricating different structures remain the main drawbacks to the widespread use of PECs. Preparing PECs in a high-throughput manner, under reproducible conditions is one the main challenges to be pursuit in the near future. The control of all the different parameters that are known to influence PEC formation is essential to keep reproducibility among different PEC batches and will require a synergy between different expertise and knowledge fields, from chemistry to robotics, to physics, to nanotechnology, to biomaterials engineering, to fundamental biology, among others. Only through the combination of the different areas, a real effective and standardized method of producing Ch/γ-PGA PEC can be reached and widespread their use within tissue engineering and regenerative medicine approaches.

ACKNOWLEDGMENTS

This work is a result of the project NORTE-01-0145-FEDER-000012 , supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). The work was also financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020

e-ISSN: 2321-6212
p-ISSN: 2347-2278

- Operational Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274). Raquel Gonçalves is also grateful to FCT for her starting grant (IF/00638/2014).

REFERENCES

1. Berger J, et al. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm 2004;57:35-52.
2. Koetting MC, et al. Stimulus-responsive hydrogels: Theory, modern advances, and applications. Mater Sci Eng R-Rep 2015;93:1-49.
3. Monge C, et al. Spatio-Temporal Control of LbL Films for Biomedical Applications: From 2D to 3D. Adv Healthc Mater 2015;4:811-830.
4. Stuart MAC, et al. Emerging applications of stimuli-responsive polymer materials. Nat Mater 2010;9:101-113.
5. Delcea M, et al. Stimuli-responsive LbL capsules and nanoshells for drug delivery. Adv Drug Del Rev 2011;63:730-747.
6. Hellmers F, et al. Characterization and in vitro cytotoxicity of doxorubicin-loaded gamma-polyglutamic acid-chitosan composite nanoparticles. Biochem Eng J 2013;75:72-78.
7. Hsieh CY, et al. Preparation of gamma-PGA/chitosan composite tissue engineering matrices. Biomaterials 2005;26:5617-5623.
8. Kang HS, et al. Polyelectrolyte complex hydrogel composed of chitosan and poly(gamma-glutamic acid) for biological application: preparation, physical properties, and cytocompatibility. J Appl Polym Sci 2007;103:386-394.
9. Liao Z-X, et al. Enhancement of efficiencies of the cellular uptake and gene silencing of chitosan/siRNA complexes via the inclusion of a negatively charged poly(γ-glutamic acid). Biomaterials 2010;31:8780-8788.
10. Tsao CT, et al. Antibacterial activity and biocompatibility of a chitosan–γ-poly(glutamic acid) polyelectrolyte complex hydrogel. Carbohydr Res 2010;345:1774-1780.
11. Chang H-H, et al. A novel chitosan-gamma PGA polyelectrolyte complex hydrogel promotes early new bone formation in the alveolar socket following tooth extraction. PLos ONE 2014;9: e92362.
12. Tsao CT, et al. Evaluation of chitosan/-poly(glutamic acid) polyelectrolyte complex for wound dressing materials. Carbohydr Polym 2011;84:812-819.
13. Lin YH, et al. Novel nanoparticles for oral insulin delivery via the paracellular pathway. Nanotechnology 2007;18:105102.
14. Sonaje K, et al. Biodistribution, pharmacodynamics and pharmacokinetics of insulin analogues in a rat model: oral delivery using pH-Responsive nanoparticles vs. subcutaneous injection. Biomaterials 2010;31:6849-6858.
15. Sonaje K, et al. In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials 2009;30:2329-2339.
16. Tang D-W, et al. Heparinized chitosan/poly(γ-glutamic acid) nanoparticles for multi-functional delivery of fibroblast growth factor and heparin. Biomaterials 2010;31:9320-9332.
17. Tang D-W, et al. Characterization of tea catechins-loaded nanoparticles prepared from chitosan and an edible polypeptide. Food Hydrocoll 2013;30:33-41.
18. Liu Y, et al. Preparation and evaluation of lysozyme-loaded nanoparticles coated with poly-gamma-glutamic acid and chitosan. Int J Biol Macromol 2013;59:201-207.
19. Nair LS and Laurencin CT. Biodegradable polymers as biomaterials. Prog Polym Sci 2007;32:762-798.
20. Rinaudo M. Chitin and chitosan: Properties and applications. Prog Polym Sci 2006;31:603-632.
21. Suh JKF and Matthew HWT. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials 2006;21:2589-2598.
22. Mansouri S, et al. Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur J Pharm Biopharm 2004;57:1-8.
23. Mao HQ, et al. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release 2001;70:399-421.
24. Mao S, et al. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev 2010;62:12-27.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

25. Richard I, et al. Ionization Behavior of Chitosan and Chitosan-DNA Polyplexes Indicate That Chitosan Has a Similar Capability to Induce a Proton-Sponge Effect as PEI. Biomacromolecules 2013;14:1732-1740.

26. Prescott AG, et al. Method for producing medical and commercial grade poly-gamma-glutamic acid of high molecular weight. 2005; US 2005/0095679 A1.

27. Prodhomme EJF, et al. Multivalent conjugates of poly-gamma-D-glutamic acid from Bacillus licheniformis with antibody F(ab') and glycopeptide ligands. Bioconjugate Chem 2003;14:1148-1155.

28. Sutherland MD, et al. In vivo fate and distribution of poly-gamma-D-glutamic acid, the capsular antigen from Bacillus anthracis. Infect Immun 2008;76:899-906.

29. Buescher JM, et al. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Crit Rev Biotechnol 2007;27:1-19.

30. Shih IL, et al. Biomedical applications of chemically and microbiologically synthesized poly(glutamic acid) and poly(lysine). Mini-Rev Med Chem 2004;4:179-188.

31. Hinoi E, et al. Glutamate signaling in peripheral tissues. Eur J Biochem 2004;271:1-13.

32. Michaelis EK. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol 1998;54:369-415.

33. Piepoli T, et al. Glutamate signaling in chondrocytes and the potential involvement of NMDA receptors in cell proliferation and inflammatory gene expression. Osteoarthr Cartil 2009;17:1076-1083.

34. Salter DM, et al. NMDA receptor expression and roles in human articular chondrocyte mechanotransduction. Biorheology 2004;41:273-281.

35. Skerry TM, et al. Glutamate signalling in non-neuronal tissues. Trends Pharmacol Sci 2001;22:174-181.

36. Frigerio F, et al. Tissue specificity of mitochondrial glutamate pathways and the control of metabolic homeostasis. Biochim Biophys Acta – Bioenergetics 2008;1777:965-972.

37. Ashiuchi M, et al. A poly-gamma-glutamate synthetic system of Bacillus subtilis IFO 3336: Gene cloning and biochemical analysis of poly-gamma-glutamate produced by Escherichia coli clone cells. Biochem Biophys Res Commun 1999;263:6-12.

38. Pereira CL, et al. Biosynthesis of highly pure poly-gamma-glutamic acid for biomedical applications. J Mater Sci Mater Med 2012;23:1583-1591.

39. Kotz J, et al. Self-assembled polyelectrolyte systems. Prog Polym Sci 2001;26:1199-1232.

40. Michaels AS. Polyelectrolyte complexes. I & EC 1965;57:32-40.

41. Ariga K, et al. Layer-by-layer nanoarchitectonics: invention, innovation, and evolution. Chem Lett 2014;43:36-68.

42. Boudou T, et al. Multiple functionalities of polyelectrolyte multilayer films: new biomedical applications. Adv Mater 2010;22:441-467.

43. Crouzier T, et al. Polysaccharide-based polyelectrolyte multilayers. Curr Opin Colloid Interface Sci 2010;15:417-426.

44. de Villiers MM, et al. Introduction to nanocoatings produced by layer-by-layer (LbL) self-assembly. Adv Drug Deliv Rev 2011;63:701-715.

45. Deshmukh PK, et al. Stimuli-sensitive layer-by-layer (LbL) self-assembly systems: targeting and biosensory applications. J Control Release 2013;166:294-306.

46. Ikkala O, et al. Hierarchical self-assembly in polymeric complexes: towards functional materials. Chem Commun (Camb) 2004;60:2131-2137.

47. Liu ZH, et al. Polysaccharides-based nanoparticles as drug delivery systems. Adv Drug Deliv Rev 2008;60:1650-1662.

48. Tang Y, et al. Physical hydrogels with self-assembled nanostructures as drug delivery systems. Expert Opin Drug Deliv 2011;8:1141-1159.

49. Barbosa MA, et al. Polysaccharides as scaffolds for bone regeneration. ITBM-RBM 2005;26:212-217.

50. Kumar M, et al. Chitosan chemistry and pharmaceutical perspectives. Chem Rev 2004;104:6017-6084.

51. Yi HM, Wu LQ, et al. Biofabrication with chitosan. Biomacromolecules 2005;6:2881-2894.

52. Sarmento B, et al. Preface. In Chitosan-Based Systems for Biopharmaceuticals. John Wiley & Sons, Ltd. 2012.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

53. Shukla SK, et al. Chitosan-based nanomaterials: a state-of-the-art review. Int J Biol Macromol 2013;59:46-58.

54. Barbosa MA, et al. Chitosan. In Comprehensive Biomaterials. Elsevier Ltd. 2011.

55. Rinaudo M, et al. Main properties and current applications of some polysaccharides as biomaterials. Polym Int 2008;57:397-430.

56. Yeul VS and Rayalu SS. Unprecedented Chitin and Chitosan: a Chemical Overview. J Polym Environ 2013;21:606-614.

57. Muzzarelli RAA and Muzzarelli C. Chitosan chemistry: relevance to the biomedical sciences. Adv Polym Sci 2005;186:151-209.

58. Prestwich GD and Atzet S. Engineering Natural Materials. In Biomaterials Science. (3rd ed), Academic Press, Oxford. 2012.

59. Roberts GAF. Chitin Chemistry. Palgrave Macmillan 1992.

60. Mourya VK and Inamdar NN. Chitosan-modifications and applications: opportunities galore. React Funct Polym 2008;68:1013-1051.

61. Dornish M, et al. Regulatory Status of Chitosan and Derivatives. In Chitosan-Based Systems for Biopharmaceuticals. John Wiley & Sons, Ltd. 2012.

62. Park JW, et al. Acid-base equilibria and related properties of chitosan. Bull Korean Chem Soc 1983;4:68-72.

63. Luo K, et al. Biodegradable interpolyelectrolyte complexes based on methoxy poly(ethylene glycol)-b-poly(alpha,L-glutamic acid) and chitosan. Biomacromolecules 2008;9:2653-2661.

64. Berger J, et al. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm 2004;57:19-34.

65. Jayakumar R, et al. Biomedical applications of chitin and chitosan based nanomaterials-a short review. Carbohydr Polym 2010;82:227-232.

66. Shelke NB, et al. Polysaccharide biomaterials for drug delivery and regenerative engineering. Polym Adv Technol 2014;25:448-460.

67. Amaral IF, et al. Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation. J Biomater Sci Polym Ed 2007;18:469-485.

68. Costa F, et al. Characterization of hLF1-11 immobilization onto chitosan ultrathin films, and its effects on antimicrobial activity. Acta Biomater 2014;10:3513-3521.

69. Kang X, et al. Glucose oxidase-graphene-chitosan modified electrode for direct electrochemistry and glucose sensing. Biosens Bioelectron 2009;25:901-905.

70. Samuels RJ. Solid-state characterization of the structure of chitosan films. J Polym Sci B Polym Phys 1981;19:1081-1105.

71. Antunes JC, et al. Layer-by-layer self-assembly of chitosan and poly(gamma-glutamic acid) into polyelectrolyte complexes. Biomacromolecules 2011;12:4183-4195.

72. Chua PH, et al. Surface functionalization of titanium with hyaluronic acid/chitosan polyelectrolyte multilayers and RGD for promoting osteoblast functions and inhibiting bacterial adhesion. Biomaterials 2008;29:1412-1421.

73. Schneider A, et al. Multifunctional polyelectrolyte multilayer films: Combining mechanical resistance, biodegradability, and bioactivity. Biomacromolecules 2007;8:139-145.

74. Thierry B, et al. Bioactive coatings of endovascular stents based on polyelectrolyte multilayers. Biomacromolecules 2003;4:1564-1571.

75. Yi HM, et al. A robust technique for assembly of nucleic acid hybridization chips based on electrochemically templated chitosan. Anal Chem 2004;76:365-372.

76. Calvo P, et al. Chitosan and chitosan ethylene oxide propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharmaceut Res 1997;14:1431-1436.

77. Oliveira H, et al. Chitosan-based gene delivery vectors targeted to the peripheral nervous system. J Biomed Mater Res A 2010;95A:801-810.

78. Pires LR, et al. Imidazole-grafted chitosan-mediated gene delivery: in vitro study on transfection, intracellular trafficking and degradation. Nanomedicine 2011;6:1499-1512.

79. Roy K, et al. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Med 1999;5:387-391.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

80. Sarmento B, et al. Alginate/chitosan nanoparticles are effective for oral insulin delivery. Pharmaceut Res 2007;24:2198-2206.

81. Goncalves IC, et al. Bacterial-binding chitosan microspheres for gastric infection treatment and prevention. Acta Biomater 2013;9:9370-9378.

82. He P, et al. Chitosan microspheres prepared by spray drying. Int J Pharm 1999;187:53-65.

83. Ko JA, et al. Preparation and characterization of chitosan microparticles intended for controlled drug delivery. Int J Pharm 2002;249:165-174.

84. Berth G, et al. Polyelectrolyte complexes and layer-by-layer capsules from chitosan/chitosan sulfate. Biomacromolecules 2002;3:579-590.

85. Yan S, et al. Layer-by-layer assembly of poly(L-glutamic acid)/chitosan microcapsules for high loading and sustained release of 5-fluorouracil. Eur J Pharm Biopharm 2011;78:336-345.

86. Ye SQ, et al. New loading process and release properties of insulin from polysaccharide microcapsules fabricated through layer-by-layer assembly. J Control Release 2006;112:79-87.

87. Dash BC, et al. The influence of size and charge of chitosan/polyglutamic acid hollow spheres on cellular internalization, viability and blood compatibility. Biomaterials 2010;31:8188-8197.

88. Hu Y, et al. Core-template-free strategy for preparing hollow nanospheres. Adv Mater 2004;16:933-937.

89. Itoh Y, et al. Preparation of biodegradable hollow nanocapsules by silica template method. Chem Lett 2004;33:1552-1553.

90. Amaral IF, et al. Fibronectin-mediated endothelialisation of chitosan porous matrices. Biomaterials 2009;30:5465-5475.

91. Barbosa JN, et al. Evaluation of the effect of the degree of acetylation on the inflammatory response to 3D porous chitosan scaffolds. J Biomed Mater Res A 2010;93A:20-28.

92. Ho MH, et al. Preparation of porous scaffolds by using freeze-extraction and freeze-gelation methods. Biomaterials 2004;25:129-138.

93. Oliveira SM, et al. Engineering endochondral bone: in vivo studies. Tissue Eng Part A 2009;15:635-643.

94. Park YJ, et al. Controlled release of platelet-derived growth factor-BB from chondroitin sulfate-chitosan sponge for guided bone regeneration. J Control Release 2000;67:385-394.

95. Vasconcelos DP, et al. Macrophage polarization following chitosan implantation. Biomaterials 2013;34:9952-9959.

96. Bhattarai N, et al. Electrospun chitosan-based nanofibers and their cellular compatibility. Biomaterials 2005;26:6176-6184.

97. De Vrieze S, et al. Electrospinning of chitosan nanofibrous structures: feasibility study. J Mater Sci 2007;42:8029-8034.

98. Hirano S, et al. Wet spun chitosan-collagen fibers, their chemical N-modifications, and blood compatibility. Biomaterials 2000;21:997-1003.

99. Shao X and Hunter CJ. Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells. J Biomed Mater Res A 2007;82A:701-710.

100. Tuzlakoglu K, et al. Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications. Macromol Biosci 2004;4:811-819.

101. Yudin VE, et al. Wet spinning of fibers made of chitosan and chitin nanofibrils. Carbohydr Polym 2014;108:176-182.

102. Bhattarai N, et al. PEG-grafted chitosan as an injectable thermosensitive hydrogel for sustained protein release. J Control Release 2005;103:609-624.

103. Chen SC, et al. A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release 2004;96:285-300.

104. Crompton KE, et al. Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering. Biomaterials 2007;28:441-449.

105. Lee JW, et al. Synthesis and characteristics of interpenetrating polymer network hydrogel composed of chitosan and poly(acrylic acid). J Appl Polym Sci 1999;73:113-120.

106. Ruel-Gariepy E, et al. Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. Int J Pharm 2000;203:89-98.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

107. Kean T and Thanou M. Biodegradation, biodistribution and toxicity of chitosan. Adv Drug Deliv Rev 2010;62:3-11.

108. Dumoulin M, et al. Human Lysozyme. In Protein Misfolding, Aggregation, and Conformational Diseases. Springer US 2007.

109. Chavan SB and Deshpande MV. Chitinolytic enzymes: an appraisal as a product of commercial potential. Biotechnol Prog 2013;29:833-846.

110. Duskova J, et al. Chitinolytic enzymes from bacterium inhabiting human gastrointestinal tract - critical parameters of protein isolation from anaerobic culture. Acta Biochim Pol 2011;58:261-263.

111. Rao SB and Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 1997;34:21-28.

112. He P, et al. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. Int J Pharm 1998;166:75-88.

113. Lehr CM, et al. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int J Pharm 1992;78:43-48.

114. Usami Y, et al. Influence of chain length of N-acetyl-D-glucosamine and D-glucosamine residues on direct and complement-mediated chemotactic activities for canine polymorphonuclear cells. Carbohydr Polym 1997;32:115-122.

115. Usami Y, et al. Migration of canine neutrophils to chitin and chitosan. J Vet Med Sci 1994;56:1215-1216.

116. Usami Y, et al. Chitin and chitosan induce migration of bovine polymorphonuclear cells. J Vet Med Sci 1994;56:761-762.

117. Usami Y, et al. Chitin and chitosan stimulate canine polymorphonuclear cells to release leukotriene B(4) and prostaglandin E(2). J Biomed Mater Res 1998;42:517-522.

118. Okamoto Y, et al. Analgesic effects of chitin and chitosan. Carbohydr Polym 2002;49:249-252.

119. No HK, et al. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol 2002;74:65-72.

120. Roller S and Covill N. The antifungal properties of chitosan in laboratory media and apple juice. Int J Food Microbiol 1999;47:67-77.

121. Tikhonov VE, et al. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-2(3)-(dodec-2-enyl) succinoyl/-derivatives. Carbohydr Polym 2006;64:66-72.

122. Malette WG, et al. Chitosan - a new hemostatic. Ann Thorac Surg 1983;36:55-58.

123. Wedmore I, et al. A special report on the chitosan-based hemostatic dressing: experience in current combat operations. J Trauma 2006;60:655-658.

124. Ivanovics G and Bruckner V. Chemical and immunologic studies on the mechanism of anthrax infection and immunity. I. The chemical structure of capsule substance of anthrax Bacilli and its identity with that of the B. mesentericus. Z Immun Exp Ther 1937;90:304–318.

125. Bovarnick M. The formation of extracellular d(-)-glutamic acid polypeptide by Bacillus subtilis. J Biol Chem 1942;145:415-424.

126. He LM, et al. Bacillus lichenformis gamma-glutamyl exopolymer: Physicochemical characterization and U(VI) interaction. Environ Sci Technol 2000;34:1694-1701.

127. Nelson D and Cox MM. Lehninger Principles of Biochemistry. (4th ed.), W. H. Frreman and Company, New York 2005.

128. Hajdu I, et al. Nanoparticles prepared by self-assembly of chitosan and poly-gamma-glutamic acid. Colloid Polym Sci 2008;286:343-350.

129. Imoto T, et al. Preparation and unique pH-responsive properties of novel biodegradable nanocapsules composed of poly(γ-glutamic acid) and chitosan as weak polyelectrolytes. Macromol Biosci 2010;10:271-277.

130. Noh Y-W, et al. Enhancement of the photostability and retention time of indocyanine green in sentinel lymph node mapping by anionic polyelectrolytes. Biomaterials 2011;32:6551-6557.

131. Serizawa T, et al. Improved alternate deposition of biodegradable naturally occurring polymers onto a quartz crystal microbalance. J Polym Sci Pol Chem 1999;37:801-804.

132. Tolentino A, et al. Complexes of polyglutamic acid and long-chain alkanoylcholines: nanoparticle formation and drug release. Int J Biol Macromol 2014;66:346-353.

133. Bajaj I and Singhal R. Poly (glutamic acid) - An emerging biopolymer of commercial interest. Bioresour Technol 2011;102:5551-5561.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

134. Ogunleye A, et al. Poly-gamma-glutamic acid: production, properties and applications. Microbiol-Sgm 2015;161:1-17.

135. Hakuba H, et al. Virus-inactivating agent useful in e.g. coating material and spray for processing materials e.g. plastics, comprises poly-gamma-glutamic acid (PGA) ion complex obtained from PGA and cationic disinfectant. WO2014126099-A1 TW201437298-A. 2014.

136. Park BJ, et al. Enhanced cellular responses of vascular endothelial cells on poly-gamma-glutamic acid/PU composite film treated with microwave-induced plasma at atmospheric pressure. Macromol Res 2011;19:537-541.

137. Barrientos H, et al. Layer-by-layer films of enzymatically synthesized poly(aniline)/bacterial poly(γ-glutamic acid) for the construction of nanocapacitors. Eur Polym J 2007;43:1672-1680.

138. Du X, et al. gamma-PGA-Coated Mesoporous Silica Nanoparticles with Covalently Attached Prodrugs for Enhanced Cellular Uptake and Intracellular GSH-Responsive Release. Adv Healthc Mater 2015;4:771-781.

139. Lin YH, et al. Preparation of nanoparticles composed of chitosan/poly-gamma-glutamic acid and evaluation of their permeability through Caco-2 cells. Biomacromolecules 2005;6:1104-1112.

140. Sano K, et al. Radiolabeled gamma-polyglutamic acid complex as a nano-platform for sentinel lymph node imaging. J Control Release 2014;194:310-315.

141. Shin DH, et al. Self-Assembled Polyelectrolyte Nanoparticles as Fluorophore-Free Contrast Agents for Multicolor Optical Imaging. Molecules 2015;20:4369-4382.

142. Sung H, et al. Composition, useful e.g. to treat diabetes, comprises nanoparticles comprising shell portion comprising positively charged chitosan, and core portion comprising positively charged chitosan, negatively charged substrate and surfactants. US8361439-B1 2013.

143. Singh MN, et al. Freeze Dried Chitosan/Poly-(Glutamic Acid) Microparticles for Intestinal Delivery of Lansoprazole. Curr Drug Deliv 2012;9:95-104.

144. Sun Y, et al. Chitosan microparticles ionically cross-linked with poly(gamma-glutamic acid) as antimicrobial peptides and nitric oxide delivery systems. Biochem Eng J 2015;95:78-85.

145. Kuo Y-C and Chung C-Y. TATVHL peptide-grafted alginate/poly(gamma-glutamic acid) scaffolds with inverted colloidal crystal topology for neuronal differentiation of iPS cells. Biomaterials 2012;33:8955-8966.

146. Park S-B, et al. Macroscopic cavities within a microporous 3-D network: a poly(gamma-glutamic acid) monolith prepared by combination of particulate templates and a phase separation technique. Polymer 2013;54:6114-6118.

147. Obata A, et al. Poly(gamma-glutamic acid)-silica hybrids with fibrous structure: effect of cation and silica concentration on molecular structure, degradation rate and tensile properties. RSC Adv 2014;4:52491-52499.

148. Wang SG, et al. Fabrication and morphology control of electrospun poly(gamma-glutamic acid) nanofibers for biomedical applications. Colloids Surf B Biointerfaces 2012;89:254-264.

149. Wang SG, et al. Poly(amidoamine) Dendrimer-Enabled Simultaneous Stabilization and Functionalization of Electrospun Poly(gamma-glutamic acid) Nanofibers. ACS Appl Mater Interfaces 2014;6:2153-2161.

150. Yoshida H, et al. Disulfide-crosslinked electrospun poly(gamma-glutamic acid) nonwovens as reduction-responsive scaffolds. Macromol Biosci 2009;9:568-574.

151. Yoshida H, et al. Scaffold-mediated 2D cellular orientations for construction of three dimensionally engineered tissues composed of oriented cells and extracellular matrices. Adv Funct Mater 2009;19:1000-1007.

152. Chen MC, et al. Poly-gamma-glutamic acid microneedles with a supporting structure design as a potential tool for transdermal delivery of insulin. Acta Biomater 2015;24:106-116.

153. Choi HJ, et al. Synthesis and characterization of pH-sensitive and biodegradable hydrogels prepared by gamma-irradiation using microbial poly(gamma-glutamic acid) and poly(epsilon-lysine). J Appl Polym Sci 1995;58:807-814.

154. Fan KS, et al. Hydrolytic and enzymatic degradation of poly(gamma-glutamic acid) hydrogels and their application in slow-release systems for proteins. J Environ Polym Degrad 1996;4:253-260.

155. Hsu S-H and Lin C-H. The properties of gelatin-poly (gamma-glutamic acid) hydrogels as biological glues. Biorheology 2007;44:17-28.

156. Murakami S and Aoki N. Bio-based hydrogels prepared by cross-linking of microbial poly(gamma-glutamic acid) with various saccharides. Biomacromolecules 2006;7:2122-2127.

157. Murakami S, et al. Bio-based biodegradable hydrogels prepared by crosslinking of microbial poly(gamma-glutamic acid)

e-ISSN: 2321-6212
p-ISSN: 2347-2278

with L-lysine in aqueous solution. Polym J 2011;43:414-420.

158. Rodriguez DE, et al. Synthesis and swelling characteristics of semi-interpenetrating polymer network hydrogels composed of poly(acrylamide) and poly(gamma-glutamic acid). Mater Lett 2006;60:1390-1393.

159. Edde B, et al. Posttranslational glutamylation of alpha-tubulin. Science 1990;247:83-85.

160. Candela T and Fouet A. Poly-gamma-glutamate in bacteria. Mol Microbiol 2006;60:1091-1098.

161. Rhie GE, et al. A dually active anthrax vaccine that confers protection against both bacilli and toxins. Proc Natl Acad Sci USA 2003;100:10925-10930.

162. Schneerson R, et al. Poly(gamma-D-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of Bacillus anthracis: a potential addition to the anthrax vaccine. Proc Natl Acad Sci USA 2003;100:8945-8950.

163. Huang M-H and Yang M-C. Swelling and biocompatibility of sodium alginate/poly(gamma-glutamic acid) hydrogels. Polym Adv Technol 2010;21:561-567.

164. Antunes JC, et al. Poly(γ-glutamic acid) as an exogenous promoter of chondrogenic differentiation of human mesenchymal stem/stromal cells. Tissue Eng Part A 2015;21:1869-1885.

165. Chang K-Y, et al. Fabrication and characterization of poly(gamma-glutamic acid)-graft-chondroitin sulfate/polycaprolactone porous scaffolds for cartilage tissue engineering. Acta Biomater 2009;5:1937-1947.

166. Lee H, et al. Effects of poly-gamma-glutamic acid on serum and brain concentrations of glutamate and GABA in diet-induced obese rats. Nutr Res Pract 2010;4:23-29.

167. Barth JV, et al. Engineering atomic and molecular nanostructures at surfaces. Nature 2005;437:671-679.

168. Cademartiri L, et al. Using shape for self-assembly. Philos Trans A Math Phys Eng Sci 2012;370:2824-2847.

169. Gazit E. Bioinspired chemistry: diversity for self-assembly. Nat Chem 2010;2:1010-1011.

170. He LZ, et al. Biomolecular engineering at interfaces. Chem Eng Sci 2006;61:989-1003.

171. Whitesides GM, et al. Molecular self-assembly and nanochemistry - a chemical strategy for the synthesis of nanostructures. Science 1991;254:1312-1319.

172. Ozin GA, et al. Nanofabrication by self-assembly. Mater Today (Kidlington) 2009;12:12-23.

173. Stupp SI and Palmer LC. Supramolecular chemistry and self-assembly in organic materials design. Chem Mater 2014;26:507-518.

174. Whitesides GM and Grzybowski B. Self-assembly at all scales. Science 2002;295:2418-2421.

175. Hill JP, et al. Self-assembly: from amphiphiles to chromophores and beyond. Molecules 2014;19:8589-8609.

176. Semino CE. Self-assembling peptides: from bio-inspired materials to bone regeneration. J Dent Res 2008;87:606-616.

177. Tamerler C and Sarikaya M. Molecular biomimetics: utilizing nature's molecular ways in practical engineering. Acta Biomater 2007;3:289-299.

178. van Rijn P. Polymer directed protein assemblies. Polymers 2013;5:576-599.

179. Butler PJG. Self-assembly of tobacco mosaic virus: the role of an intermediate aggregate in generating both specificity and speed. Philos Trans R Soc Lond B Biol Sci 1999;354:537-550.

180. Eber FJ, et al. RNA-controlled assembly of tobacco mosaic virus-derived complex structures: from nanoboomerangs to tetrapods. Nanoscale 2015;7:344-355.

181. Lebeurier G, et al. Inside-out model for self-assembly of tobacco mosaic-virus. Proc Natl Acad Sci USA 1977;74:149-153.

182. Kunitake T. Synthetic bilayer-membranes - molecular design, self-organization, and application. Angew Chem Int Ed Engl 1992;31:709-726.

183. Monnard PA and Deamer DW. Membrane self-assembly processes: steps toward the first cellular life. Anat Rec 2002;268:196-207.

184. Tien HT and Ottova AL. The lipid bilayer concept and its experimental realization: from soap bubbles, kitchen sink, to bilayer lipid membranes. J Membr Sci 2001;189:83-117.

185. Liu H, et al. Helical nanostructures based on DNA self-assembly. Nanoscale 2014;6:9331-9338.

186. Kadler KE, et al. Collagen fibril formation. Biochem J 1996;316:1-11.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

187. Mwale F. Collagen and Other Proteins of the Nucleus Pulposus, Annulus Fibrosus, and Cartilage End Plates. In The Intervertebral Disc. Springer: Wien 2014.

188. O'Leary LER, et al. Multi-hierarchical self-assembly of a collagen mimetic peptide from triple helix to nanofibre and hydrogel. Nat Chem 2011;3:821-828.

189. Wierzbicka-Patynowski I and Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. J Cell Sci 2003;116:3269-3276.

190. Hosseini MW. Molecular tectonics: from simple tectons to complex molecular networks. Acc Chem Res 2005;38:313-323.

191. Bishop KJM, et al. Nanoscale forces and their uses in self-assembly. Small 2009;5:1600-1630.

192. Whitesides GM and Boncheva M. Beyond molecules: self-assembly of mesoscopic and macroscopic components. Proc Natl Acad Sci USA 2002;99:4769-4774.

193. Betancourt T and Brannon-Peppas L. Micro- and nanofabrication methods in nanotechnological medical and pharmaceutical devices. Int J Nanomedicine 2006;1:483-495.

194. Faul CFJ, Antonietti M. Ionic self-assembly: facile synthesis of supramolecular materials. Adv Mater 2003;15:673-683.

195. Chandler D. Interfaces and the driving force of hydrophobic assembly. Nature 2005;437:640-647.

196. Cook TR, et al. Met al-organic frameworks and self-assembled supramolecular coordination complexes: comparing and contrasting the design, synthesis, and functionality of met al-organic materials. Chem Rev 2013;113:734-777.

197. Grzybowski BA, et al. Self-assembly: from crystals to cells. Soft Matter 2009;5:1110-1128.

198. Hunter CA, et al. Aromatic interactions. J Chem Soc, Perkin Trans 2001;2:651-669.

199. Mendes AC, et al. Self-assembly in nature: using the principles of nature to create complex nanobiomaterials. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2013;5:582-612.

200. Moelbert S, et al. Solvent-induced micelle formation in a hydrophobic interaction model. Phys Rev E 2004.

201. Nangia A. Conformational polymorphism in organic crystals. Acc Chem Res 2008;41:595-604.

202. Rehm T and Schmuck C. How to achieve self-assembly in polar solvents based on specific interactions? Some general guidelines. Chem Commun 2008;21:801-813.

203. Decher G. Fuzzy nanoassemblies: toward layered polymeric multicomposites. Science 1997;277:1232-1237.

204. Decher G. Layered Nanoarchitectures via Directed Assembly of Anionic and Cationic Molecules. In Templating, Self-Assembly and Self-Organization. Pergamon Press, Oxford 1996.

205. Bishop KJM, et al. When and why like-sized, oppositely charged particles assemble into diamond-like crystals. J Phys Chem Lett 2013;4:1507-1511.

206. Caruso F. Hollow capsule processing through colloidal templating and self-assembly. Chem Eur J 2000;6:413-419.

207. Faul CFJ. Ionic self-assembly for functional hierarchical nanostructured materials. Acc Chem Res 2014;47:3428-3438.

208. Michaels AS and Miekka RG. Polycation-polyanion complexes: preparation and properties of poly-(vinylbenzyltrimethykammonium) poly-(styrenesulfonate). J Phys Chem 1961;65:1765–1773.

209. Seyrek E, Decher G. Layer-by-Layer Assembly of Multifunctional Hybrid Materials and Nanoscale Devices. In Polymer Science. Elsevier BV: Amsterdam 2012.

210. Decher G and Hong JD. Buildup of ultrathin multilayer films by a self-assembly process. 1. Consecutive adsorption of anionic and cationic bipolar amphiphiles on charged surfaces. Makromol Chem, Macromol Symp 1991;46:321-327.

211. Katagiri K and Caruso F. Functionalization of colloids with robust inorganic-based lipid coatings. Macromolecules 2004;37:9947-9953.

212. Amaike M, et al. Sphere, honeycomb, regularly spaced droplet and fiber structures of polyion complexes of chitosan and gellan. Macromol Rapid Commun 1998;19:287-289.

213. Decher G, et al. Buildup of ultrathin multilayer films by a self-assembly process. 3. Consecutively alternating adsorption of anionic and cationic polyelectrolytes on charged surfaces. Thin Solid Films 1992;210:831-835.

214. Ouyang and Mueller M. Monomodal polyelectrolyte complex nanoparticles of PDADMAC/poly(styrenesulfonate): preparation and protein interaction. Macromol Biosci 2006;6:929-941.

215. Dautzenberg H, et al. Stoichiometry and structure of polyelectrolyte complex particles in diluted solutions. Ber Bunsen Phys

e-ISSN: 2321-6212
p-ISSN: 2347-2278

Chem 1996;100:1024-1032.

216. Kabanov AV, et al. Soluble stoichiometric complexes from poly(N-ethyl-4-vinylpyridinium) cations and poly(ethylene oxide)-block-polymethacrylate anions. Macromolecules 1996;29:6797-6802.

217. Solomatin SV, et al. Nanomaterials from ionic block copolymers and single-, double-, and triple-tail surfactants. Langmuir 2007;23:2838-2842.

218. Richert L, et al. pH dependent growth of poly(L-lysine)/poly(L-glutamic) acid multilayer films and their cell adhesion properties. Surf Sci 2004;570:13-29.

219. Zhao Y, et al. Self-assembled pH-responsive hydrogels composed of the RATEA16 peptide. Biomacromolecules 2008;9:1511-1518.

220. Chen S, et al. Highly active porous scaffolds of collagen and hyaluronic acid prepared by suppression of polyion complex formation. J Mater Chem B 2014;2:5612-5619.

221. Zhang J, et al. Natural polyelectrolyte films based on layer-by layer deposition of collagen and hyaluronic acid. Biomaterials 2005;26:3353-3361.

222. Kabanov AV and Kabanov VA. DNA complexes with polycations for the delivery of genetic material into cells. Bioconjug Chem 1995;6:7-20.

223. Lvov Y, et al. Assembly of thin-films by means of successive deposition of alternate layers of DNA and poly(allylamine). Macromolecules 1993;26:5396-5399.

224. Lvov Y, et al. Successive deposition of alternate layers of polyelectrolytes and a charged virus. Langmuir 1994;10:4232-4236.

225. Caruso F, et al. Magnetic core-shell particles: preparation of magnetite multilayers on polymer latex microspheres. Adv Mater 1999;11:950-953.

226. Dong H and Hinestroza JP. Metal nanoparticles on natural cellulose fibers: electrostatic assembly and in situ synthesis. ACS Appl Mater. Interfaces 2009;1:797-803.

227. Mayer ABR, et al. Colloidal silver nanoparticles protected by water-soluble nonionic polymers and "soft" polyacids. J Macromol Sci Pure Appl Chem A 1999;36:1427-1441.

228. Schmitt J, et al. Metal nanoparticle/polymer superlattice films: fabrication and control of layer structure. Adv Mater 1997;9:61-65.

229. Cademartiri L and Bishop KJM. Programmable self-assembly. Nat Mater 2015;14:2-9.

230. Caruso F, et al. Nanoengineering of inorganic and hybrid hollow spheres by colloidal templating. Science 1998;282:1111-1114.

231. Picart C, et al. Buildup mechanism for poly(L-lysine)/hyaluronic acid films onto a solid surface. Langmuir 2001;17:7414-7424.

232. Solomatin SV, et al. Environmentally responsive nanoparticles from block ionomer complexes: effects of pH and ionic strength. Langmuir 2003;19:8069-8076.

233. Tien J, et al. Microfabrication through electrostatic self-assembly. Langmuir 1997;13:5349-5355.

234. Wu H-D, et al. Chitosan-based polyelectrolyte complex scaffolds with antibacterial properties for treating dental bone defects. Mater Sci Eng C Mater Biol Appl 2012;32:207-214.

235. Yamamoto H and Senoo Y. Polyion complex fiber and capsule formed by self-assembly of chitosan and gellan at solution interfaces. Macromol Chem Phys 2000;201:84-92.

236. Tsuchida E and Abe K. Interactions between macromolecules in solution and intermacromolecular complexes. Adv Polym Sci 1982;45:1-119.

237. Vidal RRL, et al. Solution properties of partially hydrolysed polyacrylamide and chitosan mixed solutions. Macromol Symp 2005;229:118-126.

238. Biesheuvel PM and Stuart MAC. Electrostatic free energy of weakly charged macromolecules in solution and intermacromolecular complexes consisting of oppositely charged polymers. Langmuir 2004;20:2785-2791.

239. Gomes CP, et al. Translating chitosan to clinical delivery of nucleic acid-based drugs. MRS Bull 2014;39:60-70.

240. Grenha A. Chitosan nanoparticles: a survey of preparation methods. J Drug Target 2012;20:291-300.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

241. Fuoss RM. Polyelectrolytes. Science 1948;108:545-550.

242. Fuoss RM and Strauss UP. Polyelectrolytes. 2. Poly-4-vinylpyridonium chloride and poly-4-vinyl-N-N-butylpyridonium bromide. J Polym Sci 1948;3:246-263.

243. Fuoss RM and Strauss UP. Electrostatic interaction of polyelectrolytes and simple electrolytes. J Polym Sci 1948;3:602-603.

244. Michaels AS, et al. A conductometric study of polycation-polyanion reactions in dilute aqueous solution. J Phys Chem 1965;69:1447-1455.

245. Michaels AS and Morelos O. Polyelectrolyte adsorption by kaolinite. 1055; I & EC 47:1801-1809.

246. Izumrudov VA, et al. Behaviour of non-stoichiometric polyelectrolyte complexes in aqueous salt solutions. Polym Sci USSR 1980;22:767–776.

247. Kabanov VA and Zezin AB. Soluble interpolymeric complexes as a new class of synthetic polyelectrolytes. Pure Appl Chem 1984;56:343-354.

248. Zezin AB and Kabanov VA. New form of the poly-electrolytes water-soluble complexes. Usp Khim 1982;51:1447-1483.

249. Tsuchida E, et al. Formation of interpolymer complexes. J Macromol Sci Phys 1980; B17:683-714.

250. Tsuchida E, et al. Interaction of poly(styrene sulfonate) with polycations carrying charges in chain backbone. J Polym Sci Pol Chem 1972;10:3397-3404.

251. Dautzenberg H. Polyelectrolyte complex formation in highly aggregating systems. 1. Effect of salt: polyelectrolyte complex formation in the presence of NaCl. Macromolecules 1997;30:7810-7815.

252. Dautzenberg H and Karibyants N. Polyelectrolyte complex formation in highly aggregating systems. Effect of salt: response to subsequent addition of NaCl. Macromol Chem Phys 1999;200:118-125.

253. Philipp B, et al. Polyelectrolyte complexes - recent developments and open problems. Prog Polym Sci 1989;14:91-172.

254. Müller M, et al. Needlelike and spherical polyelectrolyte complex nanoparticles of poly(L-lysine) and copolymers of maleic acid. Langmuir 2005;21:465-469.

255. Thunemann AF, et al. Polyelectrolyte complexes. Adv Polym Sci 2004;166:113-171.

256. Bertrand P, et al. Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: suitable materials, structure and properties. Macromol Rapid Commun 2000;21:319-348.

257. Kirkland JJ. Porous Thin-Layer Modified Glass Bead Supports for Gas Liquid Chromatography. Anal Chem 1965;37:1458-1461.

258. Iler RK. Multilayers of colloidal particles. J Colloid Interface Sci 1996;21:569-594.

259. Decher G. Layer-by-Layer Assembly (Putting Molecules to Work). In Multilayer Thin Films. (2nd ed), Wiley-VCH Verlag GmbH & Co. KGaA., Weinheim 2012.

260. Bergna HE and Kirkland JJ. Mfg. superficially porous macro:particles - by spray drying and sintering useful as supports for chromatography and catalysts. US4477492-A EP123293-A JP59222224-A CA1215704-A EP123293-B DE3464857-G JP91051459-B 1984.

261. Kirkland JJ. Superficially porous supports for chromatography. US3505785 A 1970.

262. Fromherz P. Assembling of Proteins at Lipid Monolayers. In Electron Microscopy at Molecular Dimensions. Springer, Berlin 1980.

263. Gölander CG, et al. Heparin surface film formation through adsorption of colloidal particles studied by ellipsometry and scanning electron microscopy. Colloids Surf 1982;5:1-16.

264. Nicolau YF. Solution deposition of thin solid compound films by a successive ionic-layer adsorption and reaction process. Appl Surf Sci 1985;22-3:1061-1074.

265. Aksberg R and Ödberg L. Adsorption of an anionic polyacrylamide on cellulosic fibers with pre-adsorbed cationic polyelectrolytes. Nord Pulp Pap Res J 1990;5:168-171.

266. Decher G and Hong JD. Buildup of ultrathin multilayer films by a self-assembly process. 2. Consecutive adsorption of anionic and cationic bipolar amphiphiles and polyelectrolytes on charged surfaces. Ber Bunsen Phys Chem 1991;95:1430-1434.

267. Schönhoff M. Layered polyelectrolyte complexes: physics of formation and molecular properties. J Phys Condens Matter 2003;15: R1781-R1808.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

268. Schlenoff JB and Dubas ST. Mechanism of polyelectrolyte multilayer growth: charge overcompensation and distribution. Macromolecules 2001;34:592-598.

269. Dubas ST and Schlenoff JB. Factors controlling the growth of polyelectrolyte multilayers. Macromolecules 1999;32:8153-8160.

270. von Klitzing R. Internal structure of polyelectrolyte multilayer assemblies. Phys Chem Chem Phys 2006;8:5012-5033.

271. Brunot C, et al. Response of fibroblast activity and polyelectrolyte multilayer films coating titanium. Dent Mater 2008;24:1025-1035.

272. Dimitrova M, et al. Adenoviral gene delivery from multilayered polyelectrolyte architectures. Adv Funct Mater 2007;17:233-245.

273. Ariga K, et al. Layer-by-layer assembly as a versatile bottom-up nanofabrication technique for exploratory research and realistic application. Phys Chem Phy 2007;9:2319-2340.

274. Zhang X, Layer-by-layer assembly: from conventional to unconventional methods. Chem Commun 2007;1395-1405.

275. Cong HP and Yu SH. Self-assembly of functionalized inorganic-organic hybrids. Curr Opin Colloid Interface Sci 2009;14, 71-80.

276. Ariga K, et al. Coupling of soft technology (layer-by-layer assembly) with hard materials (mesoporous solids) to give hierarchic functional structures. Soft Matter 2009;5:3562-3571.

277. Dubas ST and Schlenoff JB. Swelling and smoothing of polyelectrolyte multilayers by salt. Langmuir 2001;17:7725-7727.

278. Jaber JA and Schlenoff JB. Dynamic viscoelasticity in polyelectrolyte multilayers: nanodamping. Chem Mater 2006;18:5768-5773.

279. Leclere P, et al. Supramolecular assembly of conjugated polymers: from molecular engineering to solid-state properties. Mater Sci Eng R Rep 2006;55:1-56.

280. Gamboa D, et al. Note: influence of rinsing and drying routines on growth of multilayer thin films using automated deposition system. Rev Sci Instrum 2010.

281. Jang WS and Grunlan JC. Robotic dipping system for layer-by-layer assembly of multifunctional thin films. Rev Sci Instrum 2005;76:1-4.

282. Fujimoto K, et al. Fabrication of layer-by-layer self-assembly films using roll-to-roll process. Appl Phys Express 2005;44: L126-L128.

283. Fu Y, et al. Facile and efficient approach to speed up layer-by-layer assembly: dipping in agitated solutions. Langmuir 2011;27:672-677.

284. Kim HJ, et al. Dynamic sequential layer-by-layer deposition method for fast and region-selective multilayer thin film fabrication. Langmuir 2005;21:8532-8538.

285. Ma L, et al. Layer-by-layer self-assembly under high gravity field. Langmuir 2012;28:9849-9856.

286. An MS and Hong JD. Consecutively spin-assembled layered nanoarchitectures of poly(sodium 4-styrene sulfonate) and poly(allylamine hydrochloride). Thin Solid Films 2006;500, 74-77.

287. Chiarelli PA, et al. Controlled fabrication of polyelectrolyte multilayer thin films using spin-assembly. Adv Mater 2001;13:1167-1171.

288. Cho J, et al. Fabrication of highly ordered multilayer films using a spin self-assembly method. Adv Mater 2001;13:1076-1078.

289. Izquierdo A, et al. Dipping versus spraying: exploring the deposition conditions for speeding up layer-by-layer assembly. Langmuir 2005;21:7558-7567.

290. Lefort M, et al. Spray-on organic/inorganic films: a general method for the formation of functional nano- to microscale coatings. Angew Chem Int Ed Engl 2010;49:10110-10113.

291. Martin LJ, et al. Coating of polymers. W01999035520 A1 1999.

292. Schlenoff JB, et al. Sprayed polyelectrolyte multilayers. Langmuir 2000;16:9968-9969.

293. Hiller J, et al. Reversibly erasable nanoporous anti-reflection coatings from polyelectrolyte multilayers. Nat Mater 2002;1:59-63.

294. Wang TC, et al. Selective electroless nickel plating on polyelectrolyte multilayer platforms. Langmuir 2001;17:6610-6615.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

295. Andres CM, et al. Nanocomposite Microcontainers. Adv Mater 2012;24:4597-4600.

296. Bai Y, et al. Direct-write maskless lithography of LBL nanocomposite films and its prospects for MEMS technologies. Nanoscale 2012;4:4393-4398.

297. DeRocher JP, et al. Layer-by-layer deposition of all-nanoparticle multilayers in confined geometries. ACS Appl Mater Interfaces 2012;4:391-396.

298. Radha B, et al. Layer-by-layer assembly of a met allomesogen by dip-pen nanolithography. ACS Nano 2013;7:2602-2609.

299. Ai H, et al. Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. Cell Biochem Biophys 2003;39:23-43.

300. Picart C. Polyelectrolyte multilayer films: from physico-chemical properties to the control of cellular processes. Curr Med Chem 2008;15:685-697.

301. Tang ZY, et al. Biomedical applications of layer-by-layer assembly: from biomimetics to tissue engineering. Adv Mater 2006;18:3203-3224.

302. Gonçalves RM, et al. Mesenhymal stem cell recruitment by stromal derived factor-1 delivery systems based on chitosan/poly(gamma-glutamic acid) polyelectrolyte complexes. Eur Cells Mater 2012;23:249-261.

303. Cardoso AP, et al. An Interferon-γ-delivery system based on chitosan/poly(γ-glutamic acid) polyelectrolyte complexes modulates macrophage-derived stimulation of cancer cell invasion in vitro. Acta Biomater 2015;23:157-171.

304. Caruso F, et al. Assembly of alternating polyelectrolyte and protein multilayer films for immunosensing .2. Langmuir. 1997;13:3427-3433.

305. Ladam G, et al. In situ determination of the structural properties of initially deposited polyelectrolyte multilayers. Langmuir 2000;16:1249-1255.

306. Lavalle P, et al. Comparison of the structure of polyelectrolyte multilayer films exhibiting a linear and an exponential growth regime: An in situ atomic force microscopy study. Macromolecules 2002;35:4458-4465.

307. Losche M, et al. Detailed structure of molecularly thin polyelectrolyte multilayer films on solid substrates as revealed by neutron reflectometry. Macromolecules 1998;3:8893-8906.

308. Lutolf MP and Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005;23:47-55.

309. Mohanty AK, et al. Biofibres, biodegradable polymers and biocomposites: an overview. Macromol Mater Eng 2000;276:31-24.

310. Crouzier T, et al. Layer-by-layer films as a biomimetic reservoir for rhBMP-2 delivery: controlled differentiation of myoblasts to osteoblasts. Small 2009;5:598-608.

311. Richert L, et al. Layer by layer buildup of polysaccharide films: Physical chemistry and cellular adhesion aspects. Langmuir 2004;20:448-458.

312. Tachaboonyakiat W, et al. The influence of molecular weight over the ultrathin films of biodegradable polyion complexes between chitosan and poly(gamma-glutamic acid). Polym J 2000;32:481-485.

313. Clark SL, et al. Selective deposition in multilayer assembly: SAMs as molecular templates. Supramol Sci 1997;4:141-146.

314. Maury P, et al. Patterned self-assembled monolayers on silicon oxide prepared by nanoimprint lithography and their applications in nanofabrication. Adv Funct Mater 2005;15:451-457.

315. Ferreira L. Nanoparticles as tools to study and control stem cells. J Cell Biochem 2009;108:746-752.

316. Suri S, et al. Microparticles and Nanoparticles. In Biomaterials Science. (3rd ed.), Academic Press, Oxford 2012.

317. Whitesides GM. The 'right' size in nanobiotechnology. Nat Biotechnol 2003;21:1161-1165.

318. Lebovka NI. Aggregation of Charged Colloidal Particles. Adv Polym Sci 2014;255:57-96.

319. Müller M. Sizing, Shaping and Pharmaceutical Applications of Polyelectrolyte Complex Nanoparticles. Adv Polym Sci 2014;256:197-260.

320. Müller M, et al. Preparation and life science applications of polyelectrolyte complex nanoparticles. Curr Trends Polym Sci 2009;13:1-10.

321. Müller M, et al. Polyelectrolyte complex nanoparticles of poly(ethyleneimine) and poly(acrylic acid): preparation and applications. Polymers 2011;3:762-778.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

322. Biesheuvel PM and Stuart MAC. Cylindrical cell model for the electrostatic free energy of polyelectrolyte complexes. Langmuir 2004;20:4764-4770.

323. Dautzenberg H and Jaeger W. Effect of charge density on the formation and salt stability of polyelectrolyte complexes. Macromol Chem Phys 2002;203:2095-2102.

324. Reincke F, et al. Understanding the self-assembly of charged nanoparticles at the water/oil interface. Phys Chem Chem Phys 2006;8:3828-3835.

325. Brigger I, et al. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 2002;54:631-651.

326. des Rieux A, et al. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release 2006;116:1-27.

327. Wang JJ, et al. Recent advances of chitosan nanoparticles as drug carriers. Int J Nanomedicine 2011;6:765-774.

328. Mali KS and De Feyter S. Principles of molecular assemblies leading to molecular nanostructures. Philos Trans A Math Phys Eng Sci 2013.

329. Bressan E, et al. Nanotechnology to drive stem cell commitment. Nanomedicine 2013;8:469-486.

330. Ilie I, et al. Influence of nanomaterials on stem cell differentiation: designing an appropriate nanobiointerface. Int J Nanomedicine 2012;7:2211-2225.

331. LaVan DA, et al. Small-scale systems for in vivo drug delivery. Nat Biotechnol 2013;21:1184-1191.

332. Costalat M, et al. Reversible controlled assembly of chitosan and dextran sulfate: a new method for nanoparticle elaboration. Carbohydr Polym 2014;102:717-726.

333. Mende M, et al. Influence of the hydrophobicity of polyelectrolytes on polyelectrolyte complex formation and complex particle structure and shape. Polymers 2011;3:1363-1376.

334. Mihai M, et al. Dependency of particle sizes and colloidal stability of polyelectrolyte complex dispersions on polyanion structure and preparation mode investigated by dynamic light scattering and atomic force microscopy. J Phys Chem B 2007;111:8668-8675.

335. Schatz C, et al. Versatile and efficient formation of colloids of biopolymer-based polyelectrolyte complexes. Biomacromolecules 2004;5:1882-1892.

336. Müller M, et al. Preparation of monomodal polyelectrolyte complex nanoparticles of PDADMAC/poly(maleic acid-alt-alpha-methylstyrene) by consecutive centrifugation. Langmuir 2005;21:7044-7051.

337. Pergushov DV, et al. Water-soluble complexes of star-shaped poly(acrylic acid) with quaternized poly(4-vinylpyridine). Langmuir 2008;24:6414-6419.

338. Hajdu I, et al. Cancer cell targeting and imaging with biopolymer-based nanodevices. Int J Pharm 2013;441:234-241.

339. Gonçalves RM, et al. Macrophages response to chitosan/poly-(gamma-glutamic acid) nanoparticles carrying an anti-inflammatory drug. J Mater Sci Mat Med 2015;26:167.

340. Moon H-J, et al. Mucosal immunization with recombinant influenza hemagglutinin protein and poly gamma-glutamate/chitosan nanoparticles induces protection against highly pathogenic influenza A virus. Vet Microbiol 2012;160:277-289.

341. Su F-Y, et al. Protease inhibition and absorption enhancement by functional nanoparticles for effective oral insulin delivery. Biomaterials 2012;33:2801-2811.

342. Shi D, et al. Fabrication of Biobased Polyelectrolyte Capsules and Their Application for Glucose-Triggered Insulin Delivery. ACS Appl. Mater Interfaces 2016;8:13688-13697.

343. Zhu YF, et al. Stimuli-responsive controlled drug release from a hollow mesoporous silica sphere/polyelectrolyte multilayer core-shell structure. Angew Chem-Int Edit 2005;44:5083-5087.

344. Johnston APR, et al. Layer-by-layer engineered capsules and their applications. Curr Opin Colloid Interface Sci 2006;11:203-209.

345. Delair T. Colloidal polyelectrolyte complexes of chitosan and dextran sulfate towards versatile nanocarriers of bioactive molecules. Eur J Pharm Biopharm 2011;78:10-18.

346. Peppas NA, et al. Hydrogels in biology and medicine: From molecular principles to bionanotechnology. Adv Mater 2006;18:1345-1360.

347. Wichterle O and Lim D. Hydrophilic Gels for Biological Use. Nature 1960;185:117-118.

e-ISSN: 2321-6212
p-ISSN: 2347-2278

348. Calo E and Khutoryanskiy VV. Biomedical applications of hydrogels: A review of patents and commercial products. Eur Polym J 2015;65:252-267.

349. Hoffman AS. Hydrogels for biomedical applications. Adv Drug Del Rev 2002;54:3-12.

350. Huang G-Q, et al. Preparation and characterization of O-carboxymethyl chitosan-sodium alginate polyelectrolyte complexes. Colloid Polym Sci 2015;293:401-407.

351. Hu Y, et al. Preparation and Characterization of Alginate-yaluronic Acid-chitosan based Composite Gel Beads. Wuhan Univ. Technol.-Mat. Sci. Edit. 2015;30:1297-1303.

352. Sakiyama T, et al. Preparation of a Polyelectrolyte Complex Gel from Chitosan and Kappa-carrageenan and its pH-Sensitive Swelling. J Appl Polym Sci 1993;50:2021-2025.

353. Wang HF, et al. Studies on chitosan and poly(acrylic acid) interpolymer complex. Preparation, structure, pH-sensitivity, and salt sensitivity of complex-forming poly(acrylic acid): Chitosan semi-interpenetrating polymer network. J Appl Polym Sci 1997;65:1445-1450.

354. Yan SF, et al. Fabrication of poly(L-glutamic acid)/chitosan polyelectrolyte complex porous scaffolds for tissue engineering. J Mater Chem B 2013;1:1541-1551.

355. Granero AJ, et al. Mechanical Reinforcement of Continuous Flow Spun Polyelectrolyte Complex Fibers. Macromol Biosci 2009;9:354-360.

356. Boas M, et al. Electrospinning polyelectrolyte complexes: pH-responsive fibers. Soft Matter 2015;11:1739-1747.

357. Penchev H, et al. Novel electrospun nanofibers composed of polyelectrolyte complexes. Macromol Rapid Commun 2008;29:677-681.

358. Shen F, et al. A study on the fabrication of porous chitosan/gelatin network scaffold for tissue engineering. Polym Int 2000;49:1596-1599.

359. Spencer GJ, et al. Current perspectives on NMDA-type glutamate signalling in bone. Int J Biochem Cell Biol 2007;39:1089-1104.

360. Hunter GK, et al. Modulation of crystal-formation by bone phosphoproteins – structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J 1994;300:723-728.

361. Antunes JC, et al. Poly(γ-glutamic acid) and poly(γ-glutamic acid)-based nanocomplexes enhance type II collagen production in intervertebral disc. J Mater Sci Mat Med 2016.