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AN ECONOMICAL BIODEGRADABLE POLYMER FOR NOVEL DRUG DELIVERY APPROACHES: GELATIN

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ABSTRACT: With the advent of newer technologies, in expert opinions, nanobiotechnology is expected to revolutionize or at least significantly improve the pharmaceutical and life science market within the next 15 years. The problem associated with reproducibility prevailed, a need for an economical polymer is experienced by all the researchers so as to repeat and check the reproducibility of systems. To meet all these requirements, Gelatin comes in front as the best alternate. Gelatin (or gelatin, from Latin: gelatus = stiff, frozen) is a translucent, colorless, brittle (when dry), flavorless solid, derived from collagen obtained from various animal by-products. It is commonly used as a gelling agent in food, pharmaceuticals, photography, and cosmetic manufacturing. Substances containing gelatin or functioning in a similar way are called gelatinous. Gelatin is an irreversibly hydrolyzed form of collagen. Gelatin being biodegradable in nature is a polymer of choice for the delivery of various proteins and peptides, can be an efficient and safe vaccine adjuvant, can be used for *in vitro* delivery of immunogenic CpG oligonucleotides, can be fabricated as nanoparticles and microspheres easily, hence Gelatin can be a very promising polymer for fulfilling the future need of nanobiotechnology.

INTRODUCTION: In the development process of a nanoparticulate drug delivery system for *in vivo* application, biodegradability without toxic by-products is one of the major claims, a potential matrix molecule has to fulfill. Within the past decades, a multitude of protocols described in literature used synthetic or natural base products for the preparation of biodegradable nanoparticles.

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Instead of a complete listing of all approaches, only a selection of the most significant biodegradable nanoparticle types will be reviewed here.With regards to nanoparticles based on synthetic polymers, polylactide (PLA), polyglycolide (PLG) and poly (D, L-lactide-co-glycolide) (PLGA) nanoparticles represent the most extensively investigated ones. Further polymers discussed as promising approaches are, poly(cyanoacrylate) (PCA), poly(alkylcyano acrylate) (PACA), poly(ε-caprolactone) (PCL), and poly(ester-anhydride) (PEA) [1, 2, 3, 4]. In addition to these polymers, natural biopolymers and macromolecules such as chitosan, sodium alginate, albumin, collagen [5, 6, 7] and gelatin represent a second fundamental class of base materials for nanoparticles.

Among these, nanoparticles of proteinaceous origin, e.g. albumin, collagen and gelatin have raised specific interest. Due to their intrinsic protein structure with the high number of different accessible functional groups, they bear multiple modification opportunities for coupling of e.g. targeting-ligands, crosslinkers, and shielding substances.

In the present work, gelatin nanoparticles have been chosen as promising drug delivery system candidate. Typically, this natural biopolymer is present in other fields of our daily life. It gives gummi bears consistency and without gelatin containing icing, ingredients such as fruits would not stick to a cake. Consequently, the foodstuff industry is the major purchaser of the tonnages of gelatin that are produced every year.

However, the amount of gelatin being applied in pharmaceutical industry is not negligible, as far as capsules and ointments are concerned [6]. But also for current research in fields of delivery vehicles for the controlled release of biomolecules such as proteins and nucleotides, gelatin has generated increased interest [8]. While gelatin and the delivery systems based on this polymer are biocompatible and biodegradable without toxic degradation products [9, 10, 11], they are furthermore known for high physiological tolerance and low immunogenicity since decades [12].

However, rare ethnologically caused cases of hypersensitivity reactions in the Japanese population have been described in literature [13]. But the basically beneficial properties of gelatin contributed to its proven record of safety which is also documented by the classification as "Generally Recognized as Safe" (GRAS) excipient by the US Food and Drug Administration (FDA).

So, gelatin derivatives are even constituent of intravenously administered applications as plasma expanders (e.g. Gelafundin™, Gelafusal™) and used as sealant for vascular prostheses [14, 15, 16].

The natural sources of gelatin are animals. It is obtained by mainly acidic or alkaline, but also thermal or enzymatic degradation of the structural protein collagen.

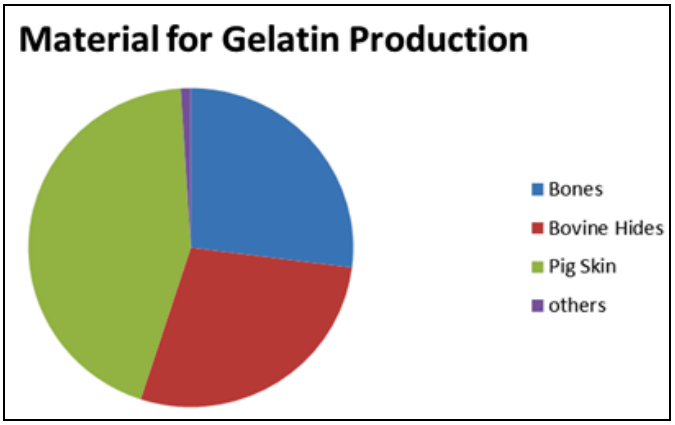

FIG. 1: MATERIALS PERCENT FOR PRODUCTION OF GELATIN

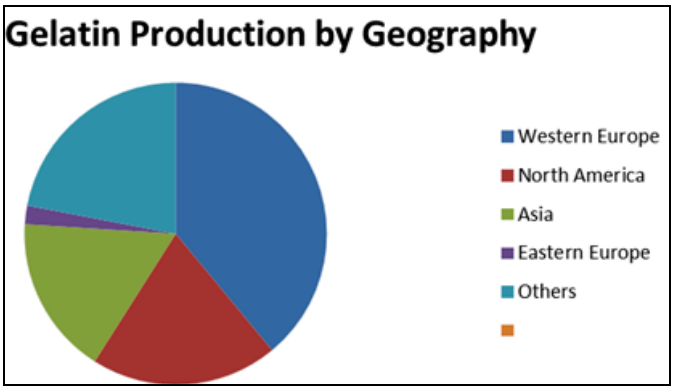

FIG. 2: PRODUCTION OF GELATIN GEOGRAPHY WISE

Collagen represents 30% of all vertebrate body protein. More than 90% of the extracellular protein in the tendon and bone and more than 50% protein in the skin consist of collagen [10]. The characteristic molecular feature of collagen being responsible for its high stability is the unique triple-helix structure consisting of three polypeptide α-chains. Among the 27 collagen types that have been isolated so far [2], only collagen type I (skin, tendon, bone), type II (hyaline vessels) and type III are utilized for the production of gelatin.

According to origin and pretreatment of the utilized collagen, two major types of gelatin are commercially produced (**Fig. 3**). Gelatin type A (acid) is obtained from porcine skin with acidic pre-treatment prior to the extraction process. The second prevalent gelatin species, type B (basic), is extracted from ossein and cut hide split from bovine origin. Thereby, an alkaline process, also known as "liming" is applied. During this extraction, also the amide groups of asparagine and glutamine are targeted and hydrolyzed into carboxyl groups, thus converting many of the residues to aspartate and glutamate [17, 18].

Consequently, the electrostatic nature is affected, in contrast to collagen and gelatin type A having an isoelectric point (IEP) of pH 9.0, the higher number of carboxyl groups per molecule reduces the IEP to pH 5.0.

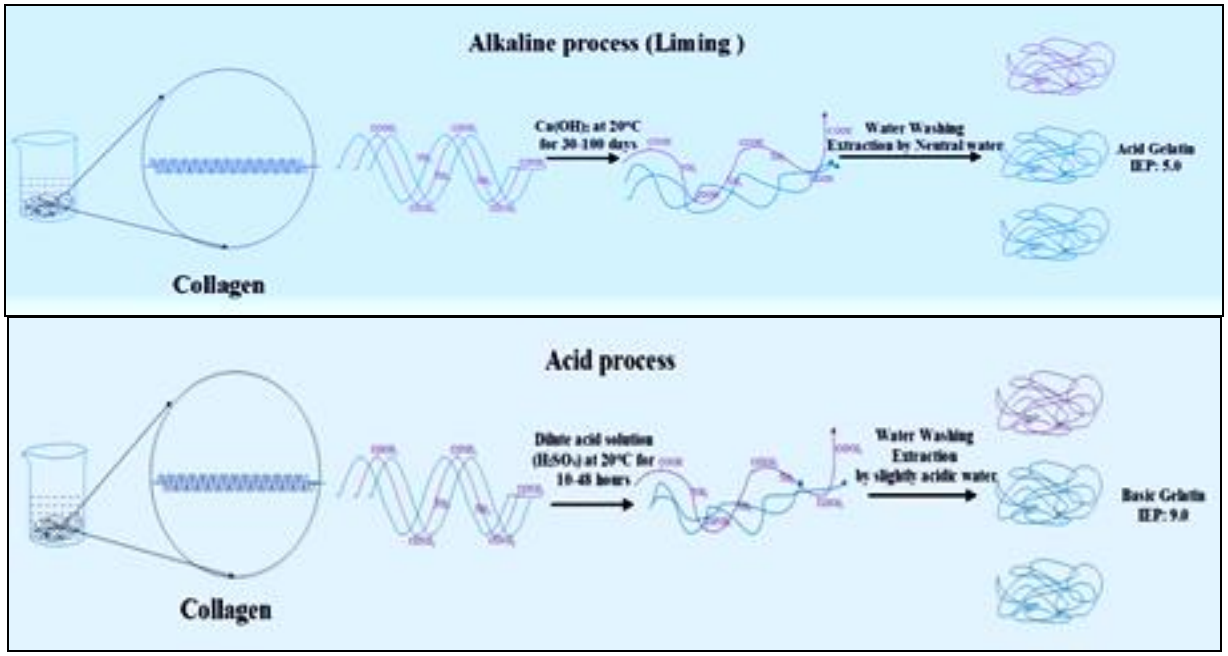

FIG. 3: PREPARATIVE PROCESS FOR ACIDIC AND BASIC GELATINS FROM

Aside from the two major gelatin types, mixtures of both, resulting in specific intermediate IEPs and cold water fish gelatin do exist. Latterly, FibroGen (South San Francisco, CA, USA) offers synthetic gelatin produced by recombinant DNA technology via a yeast system (*Pichia pastoris*). Thus, the potential theoretical hazards of animal-derived materials do no longer exist.

The amino acid composition of collagen and hence of gelatin is dominated by about 33% glycine and a further 22% proline and 4-hydroxyproline; the remaining 45% comprise 17 amino acids. This specific distribution profile can be attributed to the characteristic triple-helical structure of collagen. Thus, Gly-X-Y represents the continuously repeating amino acid sequence.

Since glycine does not possess a side chain, it is oriented into the core of the triple-helix, which represents the closest packed formation. Proline can typically be found in X-position and 4-hydroxyproline in Y-position [19]. But they can be substituted by any other amino acid as well. In addition to these repetitive sequences, there are regions of 9-26 amino acids at each end flanking the helical structure.

These non-helical regions are named telopeptides and show a high variation in amino acids. The overall amino acid composition (rounded per 1000) of type I collagen and gelatin is given in **Table 1**.

During the extraction process of collagen, covalent intra- and intermolecular bonds responsible for the stability and insolubility of collagen undergo cleavage. The resulting tropocollagen is further denatured by the breakage of hydrogen- and hydrophobic bonds that stabilize the triple-helix structure [20].

Instead of a homogeneous decomposition product, a heterogeneous proteinaceous material with a broad range of molecules with various molecular weights is generated since some peptide bonds remain stable and others, especially those with glycine are rather labile.

Furthermore, partial renaturation can occur depending on external factors such as pH or temperature [21].

The molecular heterogeneity of gelatin can be characterized via various molecular weight fractions [22].

TABLE 1: AMINO ACID COMPOSITION OF COLLAGEN TYPE I AND GELATIN (r.p. 1000)

Amino acid	Type I collagen	Type A gelatin	Type B gelatin
Alanine	113	111	117
Arginine	51	49	48
Asparagine	16	16	-
Aspartic acid	29	29	46
Glutamine	25	25	-
Glutamic acid	48	48	72
Glycine	331	329	335
Histidine	4	4	4
Hydroxylysine	104	91	93
Hydroxyproline	5	6	4
Isoleucine	11	10	11
Leucine	24	24	24
Lysine	28	27	28
Methionine	6	4	4
Phenylalanine	13	14	14
Proline	114	131	125
Serine	35	35	34
Threonine	17	18	18
Tyrosine	4	3	1
Valine	22	26	22
Total	**1000**	**1000**	**1000**

a) Low molecular weight fraction (< 50 kDa) and sub-α fraction (50-80 kDa); consisting of hydrolysis fragments.

b) α fraction (80-125 kDa); corresponding to the α-chains derived from tropocollagen.

c) β fraction (125-230 kDa); corresponding to α-chain dimers, also known as β-chain.

d) γ fraction (230-340 kDa); corresponding to α-chain trimers, also known as γ-chain.

e) ε fraction (340-700 kDa); corresponding to γ-chain dimers

f) ζ fraction (700-1000kDa); corresponding to γ-chain trimers

g) δ fraction (1000-1800 kDa); corresponding to γ-chain tetramers with a high degree of crosslinking.

h) Microgel (> 1800 kDa); corresponding to γ-chains multimers

Due to this molecular heterogeneity of gelatin, the preparation of homogeneous micro- and especially nanoparticulate formulations is challenging. Nevertheless, there is a number of preparation techniques described since the 1970s, most of them adopted from other proteins such as albumin. Basically two major approaches can be described [22, 23, 24, 25]:

a) Preparation via a biphasic system: emulsifying an aqueous solution of gelatin within an oily phase
b) Desolvation of the protein: adding a non-solvent, salting out, or adjusting the pH to the IEP of gelatin.

Most of the emulsification techniques that are described in literature use simple W/O-emulsions. Thereby, e.g. sesame oil, chloroform, toluene or isopropylpalmitate are chosen as organic phase [3, 15, 24, 25]. The preparation itself is typically performed with emulsifiers, but in some cases also without surfactant. Even though a lot of emulsion techniques are described in literature, all of them contain certain drawbacks, such as low yield (30%) or broad size distributions. Furthermore, complex and tedious purification procedures are necessary to get rid of the sometimes toxic organic phase and remaining emulsifier. Finally, high energetic methods, such as ultrasound, high-speed-, or high-pressure homogenization have to be applied to achieve adequate particle sizes.

The first method using a desolvation technique was already described in the 1970s (Speiser & Pharmaceutical Society of Victoria 1974; Marty *et al*. 1978).

Thereby, gelatin nanoparticles are prepared by the addition of sodium- or ammonium sulfate as salting-out agents or ethanol as non-solvent. This method is appropriate for the preparation of nanoparticles based on proteins with defined molecular weight such as albumin. For the use with bulk gelatin having a wide molecular weight spectrum, it lacks robustness in reproducibility and homogeneity of the produced particles. So it is described that different batches of nominally the same gelatin require different experimental conditions and even a slight excess of the dehydrating agent ethanol leads to mass aggregation and precipitation of the nanoparticles. Moreover, polydispersity indices obtained by dynamic light scattering analysis (DLS) are mostly unacceptably large, indicating the presence of a broad size distribution [25].

It is mandatory that problems like these get eliminated before gelatin nanoparticles gain attractiveness as alternative colloidal carrier system. An important step towards a solution was the development of the two-step desolvation technique [5]; a higher molecular weight fraction is separated from a lower molecular weight fraction during a first desolvation step, before the particles are prepared in a second desolvation step.

This new protocol enabled the production of homogeneous colloidal gelatin spheres. But thinking of the applicability of nanoparticles as future drug delivery systems, aside from uniformity, reproducibility in size is another prerequisite for commercial production. Hitherto, first investigations had been made, leading to a better understanding of the complexity of the process and resulting nanoparticles [5, 26].

Use of Gelatin in Delivery System:

- Gelatin Nanoparticles as biodegradable are a carrier to existing DNA delivery systems
- Gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease
- Using Gelatin as a Resveratrol Delivery Vehicle
- Gelatin-Based Nanoparticle treatment may be a more effective Clot Buster
- Gelatin Microspheres as a Multiparticulate Colonic Delivery System for 5-Aminosalicilic Acid
- Gelatin Nanoparticles as Delivery System for Nucleotide-based Drugs
- Gelatin Nanoparticle as Delivery system for treatment of ophthalmic Disorder
- Gelatin has also been used in the food industry as a stabilizer, thickener and texturizer in fruit toppings.

The therapeutic use of gelatin first appeared in 1915, when gelatin solutions were used instead of salt solutions, for the resuscitation of patients suffering from Hypovalaemia (abnormal decrease in blood volume).

Gelatins are being widely used as drug delivery vehicles in pharmaceutical applications to either deliver the drug candidate to the required therapeutic area, or to act as a carrier of the drug into the body. Gelatins have also been used in formulations of mini-pellets and tablets for protein delivery, gel formulations in combination with liposomes for sustained drug delivery, controlling material for transdermal delivery, and nanoparticles for gene delivery gelatin preparations have been used in collagen shields for ophthalmologic preparations treatment of rheumatoid arthritis, [19] sponges for burns/wounds, and as basic cell culture matrices

Gelatin has also been used for tissue engineering applications such as skin replacement and bone substitutes, and in the manufacture of artificial blood vessels and valves.

Gelatin has also been used as a drug carrier in rectal suppository formulations20 and as a delivery vehicle in protein and peptide formulations. A number of vaccine formulations that contain hydrolyzed gelatin as a component were commercially developed, including Measles, Mumps and Rubella (M.M.R), Varicella, Diphtheria and Tetanus Toxoids (DTaP) and Measles vaccines. Gelatin has also been found to be an effective lyoprotectant and stabilizer in lyophilized formulations of viral vaccines.

Characterization of Gelatin Formulations:

Particle size determination: Particle size determination can be performed with three different methods. In addition to the two state-of-the-art techniques dynamic light scattering and scanning electron microscopy, a new sizing method was evaluated. The applied new analytical tool is an asymmetric flow field-flow fractionation unit with a multiangle light scattering detector.

1. **Dynamic Light Scattering (DLS):** DLS is also often referred as photon correlation spectroscopy (PCS) or quasielastic light scattering (QELS). In DLS experiments, the Brownian motion of the analytes within the dispersion medium is detected.

   More precisely, this is done by measuring the angular distribution of time-dependent scattered light intensity due to density and/or concentration fluctuations [4]. From these fluctuations an autocorrelation function is derived, which is inverted to determine the diffusion coefficient of the analyzed sample. The diffusion coefficient in turn represents the velocity of the analyte's Brownian motion. The size of the analyte is now calculated based on the measured velocity with respect to two further factors having significant impact on this calculation; medium viscosity and temperature [27]. Thus, the hydrodynamic diameter (d(H)) can be calculated by the Stokes-Einstein equation:

$$d\,H = \frac{kT}{3\pi\eta D}$$

d(H) = hydrodynamic diameter, k = Boltzmann's constant, η = viscosity, T = absolute temperature (K), D = diffusion coefficient

Thereby, the measured diameter d(H) describes how the particles move within a liquid, but cannot be set equivalent with the actual particle diameter. The calculated DLS results are displayed as average mean sizes. However, these mean sizes represent only intensity-based average values and do not further clarify the prevailing size distributions. For this purpose a second benchmark, the polydispersity index (PI) is stated to give information about the actual distortion of the monomodal light scattering signal.

The PI can have values from 0-1 and is equivalent to the variance σ2 of the distribution [28]. DLS experiments can be performed with a Zetamaster (Malvern Instruments, Worcestershire, England) detecting the scattered light at a fixed angle of 90°. Additionally, recent samples are analyzed with a Nanosizer ZS (Malvern Instruments, Worcestershire, England) using NIBS™-technology (Non Invasive Back Scattering) at a static detection angle of 173°. Except stated otherwise, the nanoparticles are diluted in sterile filtered, highly purified water and measured in concentrations between 30 and 100 µg/mL. Due to these low concentrations, the nanoparticles did not influence the viscosity of the dispersion, so that the viscosity was set as pure water (i.e. 0.8872 cP at 25°C). The experiments should be performed at room temperature or set to 25°C in Nanosizer ZS experiments.

2. **Asymmetrical flow field-flow fractionation (AF4) in combination with multi-angle light scattering (MALS):** As novel analytical approach for size determinations is a combination of an asymmetrical flow field-flow fractionation (AF4) unit for analyte fractionation and a static light scattering (SLS) detector to determine the analyte's size or molecular weight. SLS, also known as classic light scattering measures in contrary to DLS the angular distribution of time-averaged scattered light intensities [4].

   Whereas DLS is the light scattering technique of choice, if no further fractionation of the analyte can be applied, it is inappropriate for on-line detection subsequent to a chromatographic separation, as autocorrelation and size calculation take too much time. On the other hand it is mandatory for accurate SLS detection to separate varying analytes before detection. However, SLS with a single photodiode to collect the scattered light intensity of analytes at a defined angle is not adequate for correct signal detection of gelatin nanoparticles.

   This experimental setup is only appropriate for analytes with diameters < λ/20. Solely these samples induce isotropic light scattering with the same intensity to every direction according to the Rayleigh theory.

Analytes > λ/20 instead induce anisotropic light scattering, as the light is in correlation with increasing analyte size more and more forward scattered (**Fig. 4**).

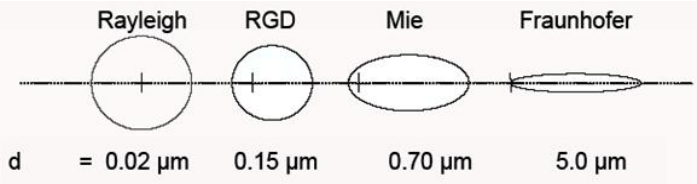

FIG. 4: ILLUSTRATION OF THE PARTICULAR LIGHT SCATTERING PROFILES OF VARIOUS SIZED ANALYTES

Hence, these analytes that are best described by the theories of Rayleigh-Gans-Debye (RGD), Mie, or Fraunhofer cannot be determined exactly by measuring only a single light intensity at a static angle. In consequence, a multi-angle measurement is necessary for correct analysis of samples within this size range. This is possible by the use of a 18-angle multi-angle light scattering (MALS) unit (DAWN EOS, Wyatt Technology, Santa Barbara, USA). For the present size calculation of gelatin nanoparticles, a Debye-based method was chosen.

The fractionation of the various sized nanoparticles was performed by an HRFFF-10.000 AF4 system, comprising separation channel, pumps controlling the injection flow, forward flow, and cross-flow, in-line solvent filter (0.1 µm, PTFE), degasser (PN7505) and autoinjection system (PN5200) (all from Postnova Analytics, Landsberg, Germany). The channel height was 350 µm and the flow rate at the channel outlet was 1.0 mL/min. The applied regenerated cellulose ultrafiltration membrane (Nadir Filtration, Wiesbaden, Germany) had a cut-off of 5 kDa.

The concentration signal, which is necessary to calculate the particle size, was obtained from on-line UV-spectrophotometrical detection at 280 nm wavelength (SpectraSystem UV 1000, Thermoquest, Darmstadt, Germany) and dn/dc values were determined with a deflection type differential RI detector (Δn-1000, λ = 620 nm; WGE Dr. Bures, Dallgow, Germany).

The experiments should be performed at 24°C, using a buffer with pH 7.4 (5 mM $Na_2HPO_4*2H_2O$ and 14 mM NaCl).

3. **Scanning Electron Microscopy (SEM):** Gelatin nanoparticles can be analyzed by SEM to characterize the surface morphology of dry, non-dispersed nanoparticles. The pictures are taken with a field emission scanning electron microscope (JSM-6500 F, Jeol, Ebersberg Germany) at 5.0 kV and a working distance of 9.7 mm. For sample preparation gelatin nanoparticles are dispersed in acetone at a concentration of 20 µg/mL and applied on a specifically polished sample grid. The samples are vacuum-dried over 12 hours and finally metallized with a 2 nm gold layer before microscopical analysis.

Molecular weight analysis of Gelatin: Size exclusion HPLC and AF4 analytics of gelatin should be performed under the technical guidance of my colleague Jan Zillies and my former colleague Dr. Wolfgang Fraunhofer.

1. **Size exclusion HPLC (SE-HPLC) analysis of various Gelatin samples:** The molecular weight distribution of dissolved gelatin type A (Bloom 175) was analyzed using a TSKgel G 3000 SW column (7.5 mm x 30 cm; Tosoh Biosep, Stuttgart, Germany). The further HPLC instrumentation consisted of a LKB 2248 pump (Pharmacia Corp., Germany), a Spectra Series AS 100 (Thermoquest, Darmstadt, Germany) autosampler, and a vacuum in-line degasser (Thermoquest). Quantitative detection was performed with a UV detector (Thermoquest) at 280nm wavelength. The light scattering signal was measured by a MiniDawn™ (Wyatt Technology, Santa Barbara, USA) static light scattering detector. The separation experiments should be performed at 24°C, using a phosphate buffer pH 6.0 (2 mM $Na_2HPO_4*2H_2O$ and 14 mM NaCl) as mobile phase. The refractive index increment dn/dc for molecular weight calculation of gelatin should be set when the second virial coefficient was set to 0.

2. **AF4 analysis of Gelatin:** The molecular weight analysis of gelatin type A (Bloom 175) can be performed with the identical AF4/MALS instrumentation as described for the nanoparticle characterization except the RI detector.

Zetapotential (ζ Potential) determination of Gelatin [29]: As the ζ potential cannot be measured directly, it is calculated from the electrophoretic mobility of an analyte, according to the Debye-Hückel-theory and the Helmholtz-Smoluchowski-equation. A NanoSizer ZS (Malvern Instruments, Worcestershire, UK) equipped with ZetaPals™ technology can be used to measure ζ potential. Measurements were performed in specific disposable cuvettes at concentrations of 20 μg/mL. To provide sufficient ionic background, measurements should be performed in 1 mM NaCl (conductivity: 0.7-1.0 mS/cm).

Visualization of formulation uptake in DCs via CLSM [30]: To enable the detection of the gelatin formulation in CLSM and FACS experiments, they are labelled with fluorescent dyes. The labelling should be conducted during the particle preparation process as shown in the **fig. 5**.

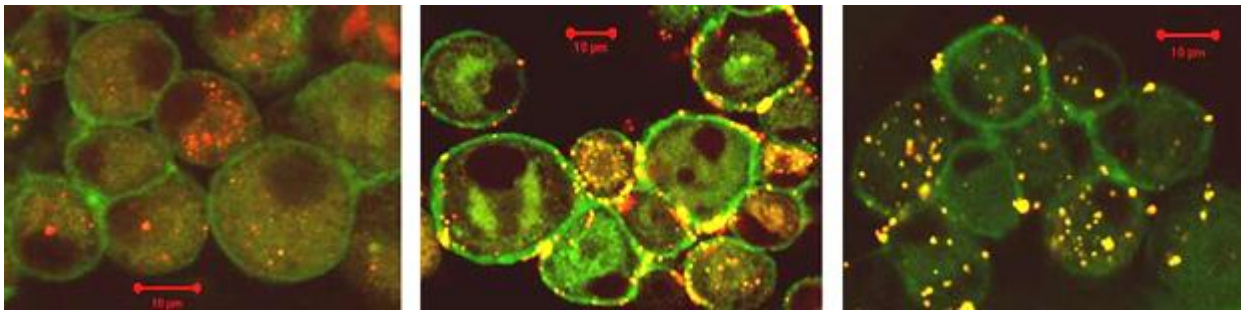

FIG. 5: CLSM PICTURES OF DCS INCUBATED WITH VARIOUS FORMULATIONS: (A) GELATIN NANOPARTICLES; (B) HSA NANOPARTICLES (C) PLGA NANOPARTICLES

Release of Protein from Gelatin: Fig. 5 shows a conceptual scheme of protein drug (IEP) of gelatin. In contrast, the electrostatic nature release from a biodegradable polymer carrier on the collagen is hardly modified through the acid basis of polyion complexation. A positively charged process because of a less invasive reaction to amide protein drug is electrostatically complexed with groups of collagen. As a result, the IEP of the gelatin negatively charged polymer chains, constituting that is obtained will remain similar to that of carrier matrix. In other words, a variety of gelatin samples increased ionic strength, occurs, the complexed drug with different IEP values are available (**Fig. 6**) will be released from the drug–carrier complex.

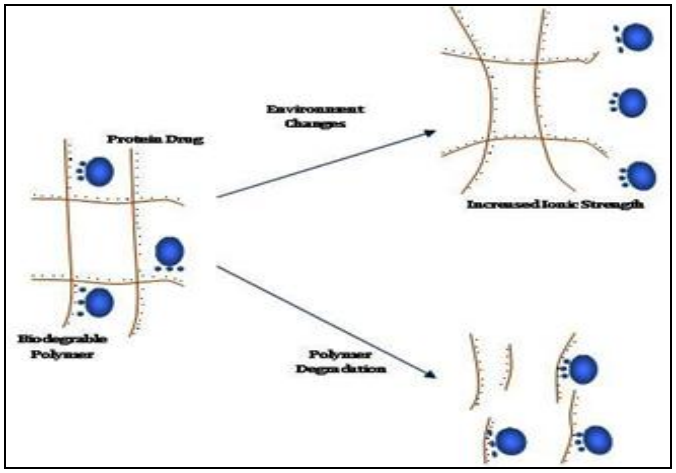

FIG. 6: RELEASE OF PROTEIN DRUG FROM BIODEGRADABLE POLYMER CARRIER ON THE BASIS OF POLYION COMPLEXATION

If a protein to be released is acidic, basic gelatin even if such an environmental change does not take with an IEP of 9.0 is preferable as the carrier place, degradation of the polymer carrier itself will material, while acidic gelatin, with an IEP of 5.0, also lead to drug release. Because the latter is more will be applicable to the sustained release of a basic likely to happen *in vivo* than the former, it is protein.

Both gelatins are insolubilized in water to preferable that the drug carrier is prepared from prepare a hydrogel through chemical crosslinking, biodegradable polymers. The profile of drug release for instance, with water-soluble carbodiimides and in this drug–carrier system is regulated by the glutaraldehyde. It was reported that a model protein change of carrier biodegradation.

CONCLUSION: Study of various aspects related to gelatin indicated the superiority of this biodegradable polymer for the use of various protein and peptide delivery. As it is very much compatible with the human system which is supported by the study of their production, can used for various formulations as section, use of gelatin nanoparticles indicates and the other characteristics can easily be analyzed by various methods.

Hence, to meet the future nanobiotechnology challenges, Gelatin as a vehicle shows its strong superiority over other available polymers.

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