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Concentration Gradients in Material Sciences: Methods to Design and Biomedical Applications

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Abstract

Concentration gradients are at the basis of many natural phenomena and hold great importance in human and plant biology. This basic phenomenon has inspired many material scientists to confer novel and original properties to materials with numerous biomedical and pharmaceutical applications such as drug discovery, controlled release of drugs, tissue engineering, orthopedic implants and time-temperature indicators, amongst others. While the generation of gradients in solution is well-described, their incorporation in solid materials remains challenging. This study reviews concepts and methods reported to generate gradients in respectively solutions and (at the surface or in) solid materials like polymer scaffolds and matrices. We also discuss how innovative materials possessing such gradients might be applied in biomedicine and pharmacy.

Keywords: chemical gradients, microfluidics, scaffolds, tablets, time-temperature indicators

1. Introduction

In numerous phenomena in nature a concentration gradient, i.e. a spatial difference in concentration of dissolved molecules ('solutes') or dispersed particles, is involved. Examples include intracellular O₂ and ATP concentration gradients.^[1] Also migration of cells often occurs in response to concentration gradients, as exemplified by e.g. the migration of leukocytes during inflammation^[2] and the migration of spermatozooids during egg fertilization^[3] or embryo development.^[4] Besides concentration gradients in solutions, in nature also concentration gradients occur in 'solid environments'; this is exemplified by the spatial differences in the degree of mineralization and amount of collagen which play a central role in the structure of tissues such as tendons, human dentine, cartilage and bones.^[5–7] Such gradients mediate between materials of widely different moduli, while possessing remarkable resistance to crack propagation and contact deformation.^[8] Taking inspiration from such biological phenomena, many synthetic materials in which concentration gradients are designed (named 'concentration gradient materials' (CGMs) in this review) are explored for biomedical applications. Most reports on CGMs mainly focus on the methods to make them, like microfluidic based approaches, without paying sufficient attention on how such concentration gradients in materials are harnessed for biomedical applications. Therefore this review has two major objectives. The first part aims to review the various technologies reported to generate concentration gradients, respectively in solutions and in solid materials; the second part aims to reveal successful biomedical applications of CGMs in e.g. the controlled release of drugs, drug screening, tissue engineering and time-temperature indicators.

2. Designing concentration gradients in solution

Currently, microfluidic technologies are the most common methods to generate concentration gradients in solution, thanks to high reproducibility, precision and ease of use.^[9–11] As **Figure 1** illustrates, by specifically designing microchannels it is possible to generate concentration gradients using adjacent streams with different concentrations of the compound of interest. By changing the length and the geometry of the microchannels it is therefore possible to modulate the mixing of the solutions thus inducing a gradient in the outlet channel of the device (**Figure 1A**).

^[12] Indeed, as shown in **Figure 1A**, solutions containing different concentration of one chemical

can be introduced from the top inlets and allowed to flow through the microchannels. When all the microchannels are recombined, a concentration gradient can be established across the outlet channel. The width (and length) of the outlet channel and the total number of the microchannels (as well as their geometry) thus determine the concentration gradient.

As discussed below, for the design of concentration gradients through microfluidic technologies, mono-phase and droplet-based methods can be distinguished.^[13] While in mono-phase methods only a liquid phase is applied into the microchannels, in droplet-based methods the microchannels are loaded with droplets dispersed in a continuous phase.

2.1. The mono-phase method

As **Figure 1A** illustrates, a concentration gradient in a solution can be prepared by a microfluidic system with a network of microchannels which shows a ‘tree structure’.^[14] Such type of network has the following advantages: (i) various fluids (containing e.g. dyes, etchants, proteins,...) can be applied in the microfluidic system, thus allowing the preparation of a variety of concentration gradients; (ii) the dimension of the concentration gradients can be tuned in the range of a few hundreds of microns (depending on the number and size of the microchannels).

However, the tree-type networks also show some drawbacks, like a large ‘footprint’, while reproducible production remains a challenge. Also, fabrication costs can rapidly increase due to the complexity of large footprints, especially with expensive materials (e.g. silicon chips). Larger footprints could also be detrimental for specific applications requiring magnetic fields or UV light irradiations which can rapidly induce engineering complications.

To improve the stability (robustness) and the resolution of concentration gradients, increasing the number of microchannels (and thus the number of dilutions of the compound applied in the microfluidic device) could be considered; though this might further enlarge the footprint. To overcome such limitations and reduce sample consumption, simpler ‘Y-shaped’ geometries have been proposed (**Figure 1B**).^[13] As an example, a microfluidic device with a Y-shaped network was used by Meier *et al.*^[15] to investigate the effect of a concentration gradient of 2,4-dichlorophenoxyacetic acid - an auxin derivative - on the growth of plant roots (**Figure 1C**); these authors prepared a ‘plant-on-a-chip’ platform in which a plant root could be inserted. The

dimension of the obtained (auxin) concentration gradient was tens of micrometers and could thus reach the single cell level.

A drawback of microfluidic devices as introduced in **Figure 1A-C** is that shear forces are present, which may affect the growth or signaling of cells exposed to the concentration gradients,^[16,17] while shear stress is generally not present in the natural micro-environment of (some) cells.^[18] To reduce potent damages to cells, microchannel systems which can produce concentration gradients without shear stress have been developed. As shown in **Figure 1D and E**, these devices can include either a membrane (**1D**) or a pressure balance system (**1E**).^[19–21] To minimize undesired fluid flows and shear stress due to pressure differences in the system, a porous membrane with high fluidic resistance but no resistance to molecular diffusion can be used. Based on the diffusion of the solutes through this membrane, a concentration gradient without shear stress can be thus obtained. For example, Abhyankar *et al.*^[22] designed a source/sink construct containing two polystyrene porous membranes with high fluidic resistance at the level of respectively the source (where the chemicals are introduced) and the cell addition port (**Figure 1D**). A concentration gradient can be created in the channel due to the presence of a sink. Atencia *et al.* reported a device without membranes but having a balanced pressure system consisting of convection units (made of one inlet and one outlet) able to spatially and temporally control a concentration gradient of chemicals in a central chamber. By controlling the flow rates in the lateral convection units a shear-free concentration gradient is produced in the chamber (**Figure 1E**).^[23]

2.2 The droplet-based method

Droplet-based microfluidic systems have been widely studied and applied in recent years (**Figure 1F**). Separated by their interface, droplets work as isolated chambers with their internal environment being separated from the surrounding solution. By changing the flow speed (or solution concentration in the two inlet channels), the concentration of the chemical compounds in each single droplet can be easily controlled. Therefore, each single droplet contains one concentration; an “array” of droplets forms a concentration “gradient”. To avoid the contamination of the droplets with the continuous phase, the oil liquid may be replaced by a gas.^[24]

Figure 1. (A) Schematic design of a concentration gradient-generating microfluidic network with a 'tree-structure'. Solutions of different concentrations of the compound of interest are introduced from the top inlets and allowed to flow through microchannels. Adapted with permission.^[14]

Copyright 2000, ACS publications. (B) Schematic diagram of a Y-shaped network. Adapted with permission.^[13] Copyright 2017, Royal Society of Chemistry. (C) Schematic representation of the microfluidic platform as used for exposing a live *Arabidopsis Thaliana* root to an auxin concentration gradient. Adapted with permission.^[15] Copyright 2010, Royal Society of Chemistry. (D) Upper and side view of a microfluidic device with membranes. The chemical stimulus of interest is added on the membrane in the source region. The molecules diffuse through the membrane and create a chemical gradient along the axial (x) direction of the channel, thanks to the presence of a sink; cells in the channel are thus exposed to the concentration gradient. Adapted with permission.^[22] Copyright 2006, Royal Society of Chemistry (E) Schematic diagram of a microfluidic device consisting of lateral convection units and a central chamber. By changing the flow rates in the lateral convection units, diffusion can be spatially and temporally controlled in the central chamber. Adapted with permission.^[23] Copyright 2009, Royal Society of Chemistry. (F) Schematic illustration of a method to generate droplet-based concentration gradients by varying the flow rate ratio between the two inlets.

3. Designing concentration gradients in solid materials

3.1 Two-dimensional concentration gradients in solid materials (2D CGMs)

Microfluidics. Microfluidic technologies are suitable as well to generate concentration gradients on the surface of solid materials in which microchannels are molded or etched. As shown in **Figure 2A**, Zhou *et al.*^[25] developed an approach to prepare a 2D NIPAM (N-isopropylacrylamide) concentration gradient on a polydimethylsiloxane (PDMS; widely used to prepare chips) surface in which microchannels were applied, this to study surface-regulated protein adsorption. First a “microfluidic concentration gradient generator” was fabricated. Subsequently an aqueous NIPAM monomer solution seeded with a photoinitiator benzophenone was simultaneously injected into the five microchannels to obtain a concentration gradient. As shown in **Figure 2A**, the junctions between the microchannels induce different dilutions, the fifth microchannel possesses the highest NIPAM concentration while the first microchannel is occupied by the lowest concentration due to minimal dilution of the NIPAM solution. Upon UV irradiation, photopolymerization of NIPAM takes place thus generating a NIPAM concentration gradient on the PDMS surface.

Using microfluidics also concentration gradients of biomolecules can be developed on the surface of solid materials. For instance, a concentration gradient of Arg-Gly-Asp (RGD) peptide was generated by Liu *et al.*^[26] on the surface of a poly(ethylene glycol) (PEG) hydrogel which was previously formed (by photo-polymerization) on a PDMS substrate; this to study RGD mediated cell adhesion at various peptide concentrations, as RGD is commonly used in tissue engineering to make cells adhere to a substrate. For this purpose, a chip with two inlets was used. The solution which contained PEGDA was injected into inlet A while the RGD solution was added into inlet B. In the microchannels the RGD solution became (differently) diluted by the PEGDA solution. Again, the microchannels merged into a ‘chamber’ where the RGD concentration gradient was formed following the photo-polymerization of the PEG hydrogel. In the study by Liu *et al.*, bone marrow derived rat mesenchymal stem cells (MSCs) were then cultured on the surface of the hydrogel. MSCs adhered very well on the hydrogel while their distribution followed the RGD concentration gradient.

Immersion. As illustrated in **Figure 2B**, ‘simple’ immersion of a material (‘substrate’) in an ‘immersion solution’ allows the formation of a concentration gradient at the surface of the material, based on chemical reactions occurring between the material surface and the immersion solution. The speed of immersion has an important influence on the obtained gradients. Another strategy is to gradually increase or decrease the concentration of the immersion solution (i.e. by changing flow rates of the injected solution in the reservoir), to decorate a surface in a graded fashion. This is a rather simple method which may be also suitable for large scale preparation.

As an example, Ueda-Yukoshi *et al.*^[27] fabricated a gradient at the surface of a poly(vinylene carbonate) (PVCa) film (casted on a glass; **Figure 2B**) by immersing the film into an aqueous solution of sodium hydroxide at a controlled speed, using a stepping motor. A longer immersion time led to more hydrolysis of poly(vinylene carbonate) (PVCa) into poly(hydroxymethylene) (PHM); a 2D PHM concentration gradient was thus obtained on the surface of the film. Aden *et al.*^[28] also applied the immersion method to obtain concentration gradients of poly(glycidyl methacrylate) (PGMA)-b-PVDMA copolymer on the surface of a silicon substrate. Therefore, the functionalizing agent was gradually added from the bottom side of substrate. During the gradual

‘climbing’ of the functionalizing agent on the glass substrate, the copolymer at the glass surface became gradually functionalized. Compared to the **Figure 2B** (using a stepping motor) which results in a discontinuous gradient, liquid climbing is suitable for the generation of continuous 2D CGMs.

Krämer *et al.* reported an immersion technique using gold nanoparticles to form protein gradients on a surface (**Figure 2C**), this to study cell-protein interactions.^[29] This was based upon a previous study from Nathan and co-workers showing that by immersion anionic gold nanoparticles could be gradually absorbed on a glass functionalized with cationic moieties^[30]. Since gold nanoparticles can easily interact with thiol moieties, they can easily bind proteins to form protein gold conjugates. As illustrated in **Figure 2C**, gold nanoparticles were first conjugated with proteins (BSA, ephrin-A5 or ephrin-B1). A glass slide, functionalized with poly-L-lysine, was placed into a beaker. Using an (HPLC) pump, the dispersion containing the gold-protein conjugates was added dropwise. The bottom of the glass slide which was exposed longer to the dispersion thus contained the highest density of gold-protein particles. A linear density gradient was formed along the slide by adding the gold dispersion at a constant flow. By changing flow profiles, authors showed that the gradient could be altered. Clearly, immersion methods are mainly used to prepare single concentration gradients while they are not suited to prepare gradients with complex patterns or multiple solutes.

Scaffold raising. A simple and original method was proposed by Wu *et al.* to produce 2D circular protein gradients (**Figure 2D**).^[31] First, a scaffold of radially-aligned PCL fibers was prepared by electrospinning. The scaffold was then placed into a well (of a 24-well plate) and a copper wire was used to lift the center of the scaffold (4 mm). A protein (BSA) solution was then gradually added to the well using a pump and the scaffold was removed from the well after the solution reached its top. As a result of the geometry and the difference of time exposure between the center and the periphery of the scaffold, a circular gradient was obtained, with lower concentrations of BSA at the center of the scaffold. Authors showed that a second gradient (with opposite direction) of bioactive proteins could be obtained on the same scaffold in the free spaces uncovered by the BSA. With this approach opposite circular gradients of two different proteins could be obtained on the same scaffold. The authors suggested that such scaffolds with protein concentration gradients could become of interest for wound healing and for applications involving cell migration.

UV light-induced immobilization. To prepare 2D CGMs with more complex patterns, UV induced radical polymerization is attractive.^[32] E.g., Wong *et al.*^[33] described a method to design gradients at the surface of an (acrylamide-based) material using grayscale masks printed at the surface to control the intensity of UV light during polymerization of acrylamide. After UV irradiation, a gradient polyacrylamide surface could be obtained. Following a similar concept, as shown in **Figure 2E**, Kloxin *et al.*^[34] used sliding masks to generate an ‘elasticity gradient’ at the surface of a hydrogel film. Therefore a photodegradable crosslinker (poly(ethylene glycol)-di-photodegradable acrylate (PEGdiPDA) was copolymerized with PEG monoacrylate (PEGA) to form a thin (0.25 mm) photodegradable hydrogel film. The degradation could be facilitated by the o-nitrobenzyl ether (NBE) moieties that reside within the PEGdiPDA monomer which undergo cleavage during UV irradiation. As shown in **Figure 2E**, while UV light irradiated the hydrogel film they were covered with an opaque plate moving at a certain speed from left to right; the right side of the film could receive irradiation while the left was untreated. As the crosslinker degrades upon irradiation, an elasticity gradient across the hydrogel surface was created.

Though masks are very efficient tools for generating gradients, they only allow to fabricate a single gradient per pattern and are not suitable to design gradients with more complex patterns.^[35] For example, to prepare multiple gradients in the same area, the mask needs to be aligned precisely. Mask alignment procedures are rather complicated and time consuming and usually impairs the generation of multi-component concentration gradient patterns.^[36]

Magnetic induction. A very original method to induce a 2D protein gradient at the surface of a scaffold was reported by Valmikinathan *et al.*, as illustrated in **Figure 2F**.^[37] Laminin was coupled to ferritin which contains an iron core in its structure. A scaffold of polycaprolactone-poly(ethylene glycol) (PCL-PEG) nanofibers, made by electrospinning, was placed on several magnets. Laminin-ferritin solutions of different concentrations were added onto four different zones (one concentration for each zone) of the PCL-PEG scaffold and could migrate thanks to the ability of ferritin to act under a magnetic field. Subsequently, the laminin-ferritin molecules were chemically crosslinked to the scaffold to obtain a gradient at the surface of the material.

pH-controlled protein adsorption. Ionov *et al.* were able to generate a gradient of proteins on a polymer brush consisting of two polyelectrolytes: polyacrylic acid (PAA) and poly2-vinyl pyridine (P2VP) (**Figure 2G**).^[38] A charge gradient was obtained by gradually changing the composition of the polymer brush. By changing the pH (from 4 to 8.6) of the protein (BSA) solution to be absorbed on the polymer brush, both the charge of BSA and the polymer brush changes (being positively charged at low pH while negatively charged at high pH). This induces variations of the electrostatic interactions between BSA and the polymer brush eventually allowing the formation of a protein gradient on the brush.

Ink-jet method (2D printing). Taking inspiration from commercial ink-jet printers, some groups have demonstrated that concentration gradients can be printed on a surface. As an example, Pardo *et al.*, have shown that patterns of BSA could be easily printed on a surface.^[39] Following the same concept, Miller *et al.* succeeded in creating protein concentration gradients by ‘overprinting’ solutions of growth factors (FGF-2 or BMP-2) on fibrin-coated surfaces, using a custom fabricated 2D printer (**Figure 2H**).^[40] After each printing session, the surface is printed again but over a shorter length (i.e. the first areas are overprinted more times than the next ones) which allows the formation of a gradient. Different types of gradients (linear or exponential) could be formed by varying the printing length and the number of overprints.

Figure 2. Summary of methods employed to generate two-dimensional concentration gradients on the surface of materials (2D CGMs). (A) Schematic representation of microchannels at the surface of a PDMS material for the generation of a concentration gradient. Depending on the length of the microchannels, the NIPAM monomer solution becomes diluted resulting in a 2D concentration gradient. Adapted with permission.^[25] Copyright 2019, Elsevier. (B) Principle of ‘top-to-bottom’ immersion. A substrate is progressively immersed into a solution containing molecules to be grafted on the surface of the substrate. The gradient is formed according to the immersion time: the longer the immersion the higher the concentration on the substrate. Adapted with permission.^[27] Copyright 1995, ACS publications. (C) A 2D protein concentration gradient on a surface can be obtained via immersion of gold nanoparticles functionalized with proteins. Adapted with permission.^[29] Copyright 2004, ACS publications. (D) Raising the center of a scaffold and flowing a protein solution allows the formation of circular protein gradients on the scaffold. Adapted with permission.^[31] Copyright, 2018 ACS publications. (E) The use of masks acting as light filters allows the generation of a 2D gradient on a surface: a sliding opaque plate allows different light exposure times which regulate the extent of a chemical reaction at the surface of a substrate, thereby leading to gradients. Adapted with permission.^[34] Copyright 2010, Elsevier. (F) Using magnets and laminin coupling to ferritin, a 2D laminin concentration gradient could be formed on a substrate. Adapted with permission.^[37] Copyright 2009, Bentham Science Publishers. (G) Formation of a protein concentration gradient on a polymer brush consisting of two (oppositely charged) polyelectrolytes. By changing the pH of the protein solution to be absorbed on the polymer brush, both the charge of BSA and the polymer brush changes allowing the formation of a protein gradient on the brush. Adapted with permission.^[38] Copyright 2009, AVS. (H) 2D-‘overprinting’ allows the formation of protein concentration gradients on substrates. Adapted with permission.^[40] Copyright 2009, Bentham Science Publishers.

3.2 Three-dimensional concentration gradients in solid materials (3D CGMs)

Microfluidics are powerful to generate gradients in 2D. Attractive as well are methods which allow to create 3-dimensional concentration gradients within the structure of polymeric matrices, like polymeric scaffolds for tissue engineering, as cells are naturally living in a three dimensional environment.

Concentration gradients made by microspheres. An original method was reported by Singh *et al.* to form a 3D concentration gradient in a cylindrical scaffold using monodisperse poly(D,L-lactide-co-glycolide) (PLG) microspheres, this for tissue engineering investigations (**Figure 3**).^[41] Two types of microspheres (respectively unloaded and loaded with a fluorescent dye) were injected with specific flow profiles into a cylindrical mold. Stacked microspheres were then washed before being soaked with ethanol to allow the spheres to attach to each other thereby forming the scaffold. Changing flow profiles, the authors showed it was possible to generate various gradients of biomolecules, being attractive to optimize drug release for tissue engineering in such scaffolds.

Figure 3. Three-dimensional concentration gradients in a scaffold obtained through the use of microspheres (loaded with a compound). Adapted with permission.^[41] Copyright 2008, Mary Ann Liebert, Inc. Publishers.

As illustrated **Figure 4A**, Peret and Murphy were able to install 3D protein concentration gradients by photo-immobilization of protein-loaded PLG microspheres into a PEG-diacrylate (PEGDA) hydrogel, this to improve three-dimensional cell culture systems.^[42] PLG microspheres loaded with rhodamine-labeled BSA were first suspended within a precursor hydrogel solution containing a photoinitiator and exposed to UV light to form a solid depot in a hydrogel (**Figure 4A**). As the proteins are released from the microspheres, they diffuse through the hydrogel thus creating a

gradient. Authors also showed that different gradients could be obtained as a function of protein size and PEGDA concentration. Clearly, while methods employing microspheres are of interest to create concentration gradients, the release of the protein from the microspheres and the incorporation of the microspheres in the scaffold need to be extensively optimized to establish optimal concentration gradients.

Immersion. As reported above, ‘immersion based methods’ allow to create 2D CGMs (**Figure 2B**). Shi *et al.* reported a similar method to create a 3D protein gradient within a scaffold consisting of electrospun nanofibers (**Figure 4B**).^[43] Nanofibers were deposited on a glass plate to make a scaffold which was subsequently positioned in a PDMS chamber; a hole was made at the bottom of the chamber to allow the injection of a protein (fibronectin) solution using a programmable syringe pump. Authors could optimize the formation of the protein gradient in the scaffold by tuning the immersion time (controlled by adjusting the filling speed of the chamber) and the concentration of the fibronectin solution.

Casting. Claussen *et al.*^[44] developed a film casting method to make gelatin films with a 3D concentration gradients of fibroin. Solutions varying in fibroin/gelatin composition were prepared. Fibroin/gelatin gradients were obtained using a syringe pump system. Syringes containing the solutions are attached to a static mixer. By applying a flow profile (i.e. by constantly changing flow rates) in the syringes, different types of mixture can be obtained. After the mixing step, the obtained protein solution was casted into a mold on a linear motion slide (**Figure 4C**). The speed of the moving mold was synchronized to the total flow rate, allowing the fibroin/gelatin solution to completely fill it to form three-dimensional films (0.2 mm thickness). Due to the difference of fibroin spatial distribution within the film, a gradient of fibroin/gelatin could be obtained. The casting method allows easy scale-up and use of organic polymers and solvents but above all, it allows the formation of concentration gradient on a large scale (up to centimeters).

Spraying. Spraying polymers and drugs is a well-known practice in pharmaceutical technology and for example applied in the coating of tablets, this to control drug release. Van Bommel *et al.*, reported on the spraying of solutions of ethylcellulose (EC), xylitol and (various amounts) of acetaminophen on the surface of a tablet made of an EC core. To form a gradient of acetaminophen

from the core to the exterior of the tablet, solutions with decreasing concentration of acetaminophen were consecutively sprayed on the EC cores (**Figure 4D**). The aim of this attractive approach was to achieve zero-order drug release and avoiding burst release.^[45] As another example, Cheng *et al.*, reported on the spraying of drug gradients on the surface of stents.^[46]

Centrifugation. Hildgen and McMullen explored centrifugation to design a 3D concentration gradient in a polymer matrix to optimize drug release from tablets.^[47] They proposed to centrifuge drug particles in a molten polymer (PCL) (**Figure 4E**). In this way a concentration gradient (increasing from the top to the bottom of the molten polymer) of respectively acetaminophen and salicylic acid could be obtained after centrifugation. Clearly, this approach requires drugs with melting points higher than that of the polymer matrix to avoid melting of the drug.

Another interesting example was reported by Roam *et al.* for the formation of scaffolds for tissue engineering.^[48] By centrifuging (drug or protein loaded) PEG microspheres of different densities they were able to form a gradient of microspheres. Since microencapsulation of drugs and proteins is well-described, this method is relatively simple and allows to obtain three dimensional gradients of different types of molecules.

3D printing. Recently, 3D printing has gained growing interest in the pharmaceutical industry, especially to fabricate multicomponent tablets (i.e. containing several drugs), this to improve patient compliance (**Figure 4F**). Moreover, this technology allows a precise customization of the dose which presents opportunities for personalized medicine.^[49] 3D printing also allows to design pharmaceutical forms in which ‘drug concentration patterns’ are present.

Haring *et al.*^[50] fabricated a 3D printed ‘polypill’ consisting of Pluronic F-127 as diluent and three drugs approved for the treatment of type 2 diabetes. Using computer-assisted printing, cylindrical pills were obtained. A custom mixing nozzle attached to multiple syringe barrels allowed real-time mixing of drug-free (Pluronic F-127 only) and drug-containing Pluronic F-127 solutions. By varying the pressure in each syringe containing the solutions, it is therefore possible to control and modulate drug concentrations in real-time to obtain a gradient after printing. This 3D printing process enables the programming of unique drug concentration profiles in the same tablet (including core–shell profile, multilayer profile and continuous gradient structures; see **Figure 4F**) which allow for different types of drug release. However, the long time required to produce such polypills (90 minutes) remains a major challenge to overcome.

Figure 4. Methods to generate three-dimensional concentration gradients within the structure of a material (3D CGM). (A) Protein-releasing PLG microspheres have been photo-immobilized in a hydrogel to produce a protein concentration gradient (left panel); the gradient as observed by fluorescence imaging (right panel; the scale bar is 1 mm). Adapted with permission.^[42] Copyright 2008, Wiley. (B) A nanofiber scaffold, made through electrospinning (left panel) is placed in a PDMS chamber having an opening at its base allowing the injection of a protein solution (middle panel). By controlling the injection speed and immersion time, a 3D protein concentration gradient can be formed in the scaffold (right panel). Adapted with permission.^[43] Copyright 2010, ACS publications. (C) The solutions containing different ratios of fibroin and gelatin are fed via a syringe pump system through a static mixer into a mold to form a gradient material. Adapted with permission.^[44,51] Copyright 2012, 2013, Wiley. (D) A tablet coated by spraying mixtures of acetaminophen/xylitol/ethylcellulose (of different ratios). The concentration of acetaminophen progressively decreases from the center to the exterior of the tablet. (E) By centrifuging drug particles in a molten polymer, a drug gradient in a matrix could be formed. Adapted with permission.^[47] Copyright 1995, Elsevier. (F) A 3D printed polypill composed of different layers; the spatial distribution of the concentration of (three) drugs varies between the layers. Adapted with permission.^[50] Copyright 2018, Wiley.

4 Biomedical and pharmaceutical applications of concentration gradient materials

4.1 Concentration gradients for optimizing tissue engineering

Cell-cell interactions, which are often mediated by concentration gradients, are considered to be of highly importance in the growth and migration of cells.^[52] As an example, concentration gradients of various substances in the microenvironment of tumors allow indirect interactions with distant cells.^[53] It explains why the induction of proper concentration gradients has been extensively studied in the context of designing organoids or tumor spheroids.^[52,54–56] In recent years, however, considerable efforts have been especially made to create concentration gradients in scaffolds that mimic the extracellular matrix (making them ‘biologically active’),^[57,58] this to engineer tissues and organs.^[59] Designing and applying concentration gradients (of growth factors)^[60,61] are indeed major challenges in the context of e.g. osteogenic and chondrogenic differentiations,^[62,63] wound healing^[64] and axon guidance (to make neurons grow their axons to reach their targets for nerve healing).^[65]

Neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 (NT-3) play a critical role in axon guidance.^[66] Shoichet and colleagues have developed a cell-adhesive and penetrable scaffold consisting poly(2-hydroxyethylmethacrylate) and poly(L-lysine) in which gradients of NGF and NT-3 were immobilized. They showed that, not only a single gradient of NGF (310 ng/ml/mm) but also lower gradients (i.e. lower concentrations) of NGF and NT-3 (200 ng/ml/mm), were efficient for neurite outgrowth in a cell-invasive scaffold (**Figure 5A**). This synergic effect allowed to get closer to the *in vivo* environment where multiple signals act together for neuron regeneration and guidance. As another example stressing the importance of gradients for neurite outgrowth, Handarmin *et al.* designed poly(ϵ -caprolactone)-poly(ethylene glycol) coaxial electrospun nanofibrous scaffolds in which a gradient of NGF was incorporated (0-250 μ g/ml).^[67] When cells were in contact with scaffolds containing the gradient, a higher percentage of cells bearing neurite outgrowth was observed, when compared with scaffolds containing a fixed NGF concentration.^[67] Also, Chang *et al.* designed a gelatin-based nerve conduit, mimicking the architecture of a nerve, with spatial gradients of neurotrophic factors (NGF and brain-derived neurotrophic factor (BDNF)) either encapsulated in gelatin nanoparticles (for controlled release) or directly incorporated in the gelatin-scaffold.^[68] *In vitro*, the neural stem cell density increased in high NGF concentration regions, while BDNF significantly promoted myelination. In a rabbit nerve section model, this type of conduit showed superior nerve recovery.

Not only the outgrowth of neurons but also the mobility and the proliferation of cells have been studied using gradients in scaffolds. For instance, by photo-immobilizing a concentration gradient of basic fibroblast growth factor (bFGF) within PEG hydrogel scaffolds, DeLong *et al.* were able to speed up the migration and proliferation of vascular smooth muscle cells (**Figure 5B**).^[69]

Methods harnessing microspheres with active substances to form concentration gradients, as discussed above, were also explored in scaffolds for tissue engineering.^[41] Wang *et al.* compared the growth of human bone marrow-derived mesenchymal stem cells (hMSCs) by applying concentration gradients in different scaffolds (either silk or alginate) using different types of microspheres (made from either PLGA or silk) loaded with bone morphogenetic protein 2 (rhBMP-2) and insulin-like growth factor I (rhIGF-I) which are known to induce osteogenic and chondrogenic differentiation.^[62] As illustrated in **Figure 5C**, by seeding cells on the silk scaffolds containing the growth factor-loaded silk microspheres, the authors showed that osteogenic and

chondrogenic differentiations occurred along the concentration gradients of rhBMP-2 and reverse dual gradients of rhBMP-2/rhIGF-I (i.e. in opposite directions) but not in presence of microspheres containing rhIGF-I showing silk microspheres were more efficient to deliver rhBMP-2 than rhIGF-I. This study illustrates the importance of the release properties of the microspheres that need to be optimized to achieve efficient cell distribution and differentiation.

Another study reported on concentration gradients to guide endothelial cells in porous collagen scaffolds.^[70] Cell death often occurs within 3D scaffolds due to a lack of oxygen and nutrients at their center which results in a necrotic core. By using survival/growth factors (e.g. vascular endothelial growth factor (VEGF)) it is possible to limit cell death. The authors of this study therefore proposed to immobilize a concentration gradient of VEGF from the periphery to the center of collagen scaffolds, this to compensate the lack of oxygen (and therefore avoid cell death) in the center of the scaffold and to allow cell migration towards the core (**Figure 5D**). Compared with VEGF-free controls and uniformly immobilized VEGF scaffolds, VEGF gradient scaffolds showed a significantly improved migration of endothelial cells in their cores.

Figure 5. Concentration gradients for optimizing tissue engineering. (A) Applying concentration gradients of growth factors (NGF and NT-3) in a polymer scaffold allows axone guidance and outgrowth. Adapted with permission.^[66] Copyright 2006, Mary Ann Liebert, Inc. publishers. (B) Photo-immobilizing concentration gradients of basic fibroblast growth factor (bFGF) in a PEG-hydrogel scaffold speed up migration and proliferation of vascular smooth muscle cells Adapted with permission.^[69] Copyright 2005, Elsevier. (C) Applying concentration gradients through the release of growth factors from microspheres in scaffolds induces osteogenic and chondrogenic differentiations of human bone marrow-derived mesenchymal stem cells (hMSCs). Adapted with permission.^[62] Copyright 2009, Elsevier. (D) Creating a VEGF gradient from the periphery to the center of a collagen scaffold improves cell viability and compensate lack of oxygen in its center and leads to improved cell migration. Adapted with permission.^[70] Copyright 2011, Elsevier.

4.2 Concentration gradients for optimizing drug release

Drug release from dosage forms (like tablets) often relies on a concentration gradient existing between the (inner part of) the dosage form and the external medium like e.g. gastric or intestinal fluids. Following oral intake, the kinetic of drug release from the dosage form strongly influences the pharmacokinetics of the drug. Achieving so named ‘zero-order release’ of a drug from its dosage form, i.e. release at a constant rate in time, is often a major goal as it allows a better control over the drug plasma levels and therefore reduced effects. While various strategies are explored, some groups suggested that applying a drug concentration gradient in the dosage form could be an attractive approach to achieve zero-order release.^[71] As one example, Van Bommel *et al.* succeeded in achieving zero-order release of acetaminophen from ethylcellulose matrices in which a drug concentration gradient was present from the core to the periphery of the matrix (**Figure 6A**).^{[45],[72]} More recently, successive applications of powder mixtures of polymer (hypromellose) and drug (acetaminophen; with a decreasing drug concentration) onto seed cores have also been reported for zero-order release kinetics.^[73] As discussed above, Haring *et al.* reported on 3D printed polypills (**Figure 4F**): this approach also allowed to achieve zero-order drug release by establishing a suitable drug concentration gradient in the pills (**Figure 6B**).^[50]

Interesting as well is that concentration gradients have also been explored to control the release of drugs from medical devices such as (drug-eluting) stents. Typically, in stents, which release drugs from their polymeric coatings (**Figure 6Ci**), burst release may occur. To avoid this Cheng *et al.*,

proposed to create a drug concentration gradient in the coating of stents through the spraying of a drug/polymer solution on the surface of the stent.^[74] This approach seemed promising to allow long-term drug release from stents without burst release (**Figure 6Cii**).

Figure 6. Applying drug concentration gradients in pharmaceutical dosage forms for zero-order release. (A) A non-uniform drug distribution (i.e. a drug concentration gradient) in non-degradable dosage forms (e.g. made from ethylcellulose) from which drug release is based on diffusion of the drug allows the ‘linearization’ of release curves. Adapted with permission.^[45] Copyright 1989, Elsevier. (B) A 3D printed pill made of three circular layers, differing in the amount of (red) dye

(as a model drug), allows zero-order release. Photographs show the pill before (gel) and after solidification (solid). Adapted with permission.^[50] Copyright 2018, Wiley. (C) Images showing uncoated stents (i) and stents coated with POSS-based thermoplastic polyurethane (POSS TPU) by spraying (ii). Applying a drug concentration gradient in the coating avoids burst drug release (iii). Adapted with permission.^{[75][74]} Copyright 2015, PLOS.

4.3 Concentration gradients for drug screening

In drug discovery, high-throughput screening is a major approach to identify new pharmacological compounds. Ye *et al.*^[76] prepared a microfluidic based device (**Figure 7A**) consisting of a drug concentration gradient generator and cell culture chambers. Authors illustrated that such a device allows to detect the effect of (the concentration) of various anticancer drugs on the plasma membrane permeability, nuclear size, mitochondrial transmembrane potential, intracellular redox states of HepG2 cells. Clearly, as this type of device produces a drug concentration gradient between only two input inlets, only two drugs can be tested at the same time. Studying the biological effect on cells of a combination of more than two drugs is therefore not suitable with this device. To solve this problem, Sweet *et al.*^[77] built a microfluidic device by 3D printing to study the combination of several drugs (**Figure 7B**). As illustrated in **Figure 7Bi and ii**, this device produces a series of concentration gradients between three drug inputs and 15 outputs; this allows to study simultaneously the biological effect of (i) different combinations of these drugs and (ii) various concentration gradients on cells. As an example, using this device the authors revealed a synergistic effect of three antibiotics (trimethoprim, nitrofurantoin, and tetracycline) on ampicillin-resistant *Escherichia coli*.

Figure 7. Concentration gradient designed for drug screening. Adapted with permission.^[76] Copyright 2007, Royal Society of Chemistry. (A) Schematic representation of an integrated microfluidic device with a network of channels that generate drug concentration gradients; cells are present in the cell culture chamber. (B) (i) The architecture of a 3D printed device which produces gradients of three drugs (A, B and C); (ii) Photo of the device. Adapted with permission.^[77] Copyright 2017, IEEE.

4.4 Concentration gradients as time and temperature indicators

Indicating time and temperature history of medication and food is of high importance to guarantee their quality, but also to avoid illegal sales of e.g. expired drugs. Indeed, environmental factors like humidity, temperature and light may influence bacterial growth and chemical degradation of food and drugs.^[78] Several systems have been designed to measure time and temperature history of consumables. Electronic devices such as radio frequency chips or data loggers can be used, however they are expensive and thus hardly applicable to every product.^[79] As a less expensive alternative, diffusion of dyes in time, which also depends on temperature, has been explored. Such devices, named ‘diffusion-type time and temperature indicators’ (TTI) are in use to confirm that products did not pass their expiration dates. Numerous TTI-patents have been filed in the last decades.^[80–84]

Suppakul *et al.* proposed a diffusion-type TTI consisting of a reservoir (filled with tween 20) linked to a microporous track strip (**Figure 8A**).^[85] Through diffusion of tween 20 in the strip a concentration gradient is built. When tween 20 reaches the test line made of poly-diacetylene/silica nanocomposite (PDA/SiO₂ NC), it changes colour irreversibly (from blue to red) indicating the TTI endpoint (i.e. the product deterioration time). Interestingly, by displacing the test line on the strip, one can easily adapt the expiration date of the TTI.

The same group proposed a strategy based on the diffusion of lactic acid into agar gels.^[86] Two pH-sensitive dyes were mixed in the agar gels (respectively methyl red and bromothymol blue; **Figure 8B**). A filter paper impregnated with lactic acid was placed on top of petri dishes containing the gels. When lactic acid diffuses from the filter paper through the gels, the pH gradually changes resulting in a progressive change of colour which was found to be dependent on time and temperature.

To track oxidation, which often affects the stability of food and drugs, technologies based on the diffusion of O₂ in a matrix or a strip have been reported. Galagan *et al.* proposed an indicator made of an oxygen-sensitive ink that can be easily placed on packages or bottles.^[87] The device consists of a substrate (where a sign can be printed) which is topped by a transparent polymer (polyacrylate) coating (**Figure 8Ci**). In the presence of O₂, the colour of the sign changes as a function of

oxidation (from red to beige) due to the presence of reduced anthraquinone in the sign (**Figure 8Cii**). To control the diffusion of the O_2 through the polymer coating, different polyacrylates varying in permeability for oxygen (which might change as a function of their glass transition temperature) were explored. The same principle has been earlier proposed by Lewis who invented a time indicator consisting of a tissue paper saturated with a reduced dye and topped with a plastic cap. As shown in **Figure 8D**, the plastic cap topology – which is thicker in its center and gradually thinner towards its periphery – allows a progressive and gradual colour change from the edges to the center as a function of oxygen diffusion over time.^[88]

Figure 8. Concentration gradients in diffusion-type time and temperature indicators (TTIs). (A) A device composed of a reservoir and track strip allows time measurements based on the change of the colour of the test line. Adapted with permission.^[85] Copyright 2018, Elsevier. (B) Diffusion of lactic acid through agar gels allows time measurements based on pH-induced colour changes. Adapted with permission.^[86] Copyright 2010, Elsevier. (C) A TTI consisting of a substrate (1), a background for printing the sign (2), a sign (3) and a transparent polymer coating (4); the diffusion of oxygen (being time and temperature dependent) through the polymer coating regulates the oxidation of the anthraquinone sign. Adapted with permission.^[87] Copyright 2008, Elsevier. (D) A polymer cap, with a thick center and a gradual decreasing thickness towards the edge, for time measurements based on the colour change of a filter paper saturated with a reduced dye. Adapted with permission.^[88]

5 Conclusions and future perspectives

This review shows how the tuning of concentration of various substances in materials can give them unique properties. It is clear from the literature that for concentration gradients in solutions at the microscale, primarily microfluidic-based approaches have been explored. However, designing concentration gradients in materials over a larger scale requires methods based on the printing, casting or immersing of compounds of interest.

As explained, there is a major interest in the potential of concentration gradients for drug screening. In this regard, important questions which remain are: how to improve the efficiency of the screening, how to reduce sample consumption and cost and how to mimic interactions between cells and drugs as close as possible to *in vivo*. As discussed as well, zero-order drug release and avoiding burst release from dosage forms and implants like stents are other objectives for which (drug) concentration gradients are under investigation. Also, concentration gradients installed through the – time and temperature dependent -diffusion of a probe in a matrix are also fully explored as time temperature indicators. This review also shows that the ‘art’ of creating concentration gradients holds great promise especially for tissue engineering. Finding optimal combinations of materials and techniques to produce these gradients remains challenging while

also a multidisciplinary approach, with contributions from various academic disciplines, will be needed to guarantee a successful use of concentration gradients in pharmacy and biomedicine.

The creation and use of concentration gradients ('chemical gradients') has been the focus of this review, with an emphasis on concentration gradients in scaffolds for tissue engineering. To optimize the 'biological performance' of scaffolds, besides designing chemical concentration gradients there is a strong interest as well in the (spatially) controlled formation of pores in scaffolds in which cells or materials can be entrapped.^[89] While 3D printing allows to 'print' drug concentration gradients in polymer scaffolds (see above), it also allows to make pores in scaffolds, with a fine control over their spatial distribution, size and shape. As an example, Sultan *et al.* could print three dimensional scaffolds with a 'pore gradient' using a hydrogel ink consisting of sodium alginate, gelatin and cellulose nanocrystals used to adapt the rheology and make the ink printable.^[90] Porous (e.g. PLGA) scaffolds were also prepared using salt particles as porogen materials;^[91] water was used to leach out the salt crystals, leaving pores behind in the scaffold.^[92] Controlling the size of the porogen particles allowed to achieve a 'pore gradient'. Similarly, Zhang *et al.*, reported on the use of ice crystals (of different size) to form pores in collagen scaffolds; a three dimensional scaffold with a gradient of micrometer-sized (150-500 μm) pores could be obtained.^[93]

Finally, this review has elaborated on the potential of 3D concentration gradients in, especially, *polymeric* solid matrices. Though, attractive as well is that chemical (and physical) gradients in metals and ceramics also find biomedical applications,^[94] like e.g. in bone grafts.^[95,96] As an example, tuning and gradually varying the porosity in metals or ceramics, through gel-casting,^[97] freeze-casting^[98] or even 3D printing, allow to better mimic the structure of bones. By taking inspiration from the graded structure of living 'interface tissues' (like tendon-to-bone and cartilage-to-bone junctions),^[99] it becomes possible to achieve 'functionally-graded' materials with new properties, when compared to homogeneous materials.^[100] A wide variety of methods have been reported to induce gradients in metals and ceramics,^[101] a topic which is however beyond the scope of this review. Though, given the attractiveness and importance of this niche of science for the future of biomaterials, we refer to Naebe *et al.* who reviewed this matter.^[101]

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